

The Expressed Genome in Cardiovascular Diseases and Stroke: Refinement, Diagnosis, and Prediction A Scientific Statement From the American Heart Association

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Abstract—There have been major advances in our knowledge of the contribution of DNA sequence variations to cardiovascular disease and stroke. However, the inner workings of the body reflect the complex interplay of factors beyond the DNA sequence, including epigenetic modifications, RNA transcripts, proteins, and metabolites, which together can be considered the “expressed genome.” The emergence of high-throughput technologies, including epigenomics, transcriptomics, proteomics, and metabolomics, is now making it possible to address the contributions of the expressed genome to cardiovascular disorders. This statement describes how the expressed genome can currently and, in the future, potentially be used to diagnose diseases and to predict who will develop diseases such as coronary artery disease, stroke, heart failure, and arrhythmias. (*Circ Cardiovasc Genet.* 2017;10:e000037. DOI: 10.1161/HCG.0000000000000037.)

Key Words: AHA Scientific Statements ■ cardiovascular diseases ■ DNA ■ epigenomics
■ genome ■ metabolomics ■ proteomics ■ stroke

In no small part as a result of the completion of the Human Genome Project, considerable effort has been invested over the past few decades in understanding the contribution of genetics to the risk of cardiovascular diseases and stroke. Genome-wide association studies, candidate gene sequencing studies, and unbiased whole-exome sequencing studies of large size have been completed for a wide variety of cardiovascular phenotypes. Many other such studies are in progress. These studies have resulted in major advances in our knowledge of the contribution of variation in the DNA sequence to these phenotypes.¹

Somewhat less attention has been given to the factors that render the genetic code into the functional consequences that influence a person’s health, that is, the “expressed genome,” including gene-regulatory elements, RNA transcripts, proteins, metabolites, and circulating cells in the bloodstream (Figure). Accordingly, the relationships between these factors and cardiovascular diseases and stroke are less well understood. The recent emergence of high-throughput technologies centered on transcriptomics, epigenomics, proteomics, metabolomics, etc, is now making it possible to address the contributions of the

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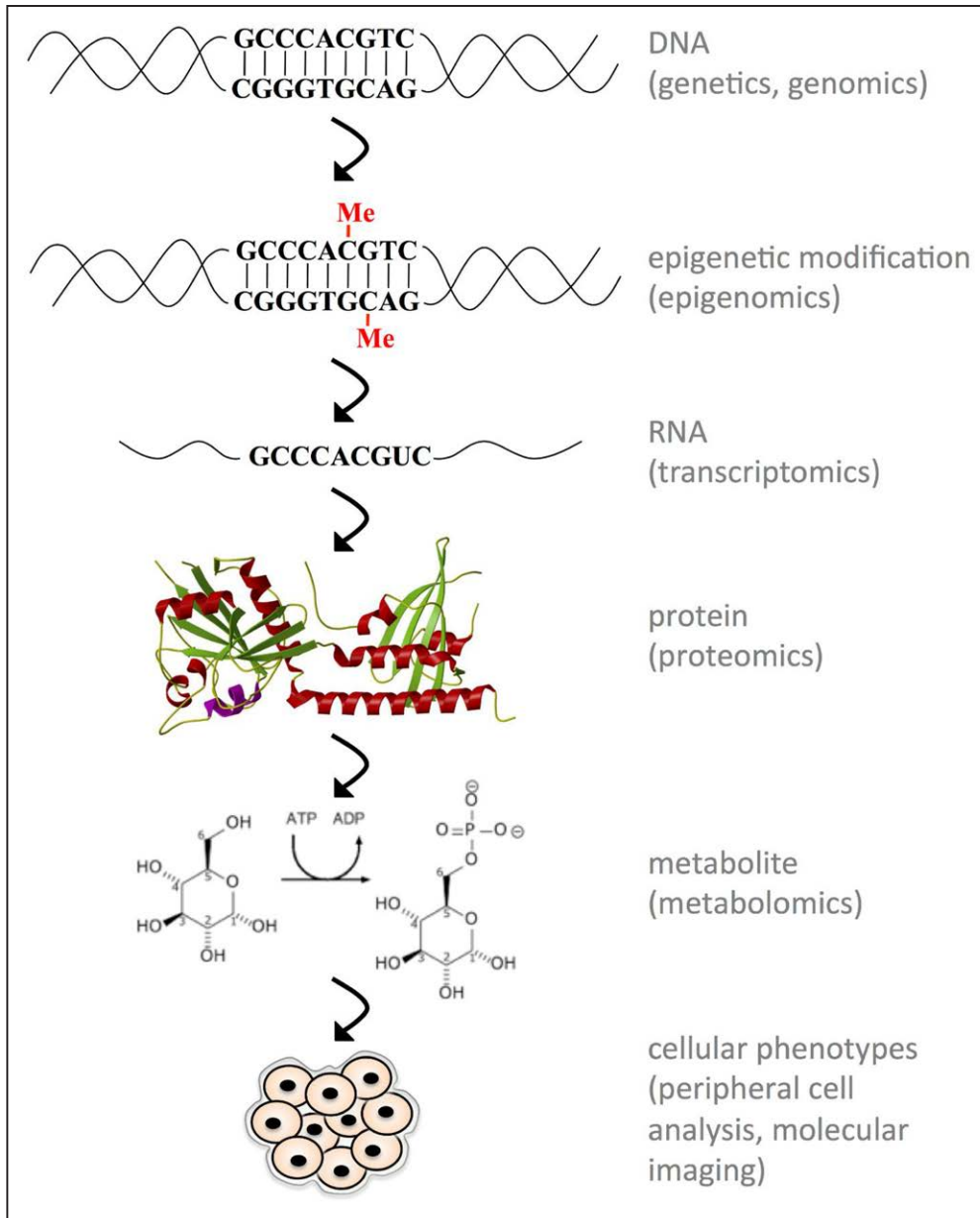


Figure. The expressed genome and associated analytic approaches. Me indicates a methyl group.

expressed genome to cardiovascular diseases and to discover novel types of biomarkers for these diseases.

The purpose of this statement is to summarize the state of the science with respect to the use of nongenetic “omics” technologies for the refinement of disease mechanisms. A major focus is coronary artery disease (CAD), but new insights into stroke, heart failure, and arrhythmias also are presented. This statement additionally serves to identify issues to be addressed to enable the use of the expressed genome for diagnosis and prediction in the clinic, especially the need for systematic replication of omics findings in independent studies.

From the Central Dogma to Whole-Organism Phenotypes

The central dogma describes the orderly transfer of genetic information from the DNA in the genome to other types

of molecules, specifically from DNA to RNA to proteins. The human genome comprises 23 pairs of chromosomes, among which are embedded an estimated 20 000 protein-coding genes. Each gene consists of a DNA sequence, with some combination of adenine, cytosine, guanine, and thymine bases, on 1 strand of the double-stranded DNA molecule that lies at the core of the chromosome. Within this DNA sequence lies the coding sequence for a protein; the coding sequence can be either a single portion of the gene sequence or multiple portions of the gene sequence that are subsequently spliced together to create a continuous coding sequence. The coding sequence contains the information needed to produce a protein.

In the process of transcription, the information in a gene sequence is transferred from the DNA strand containing the gene sequence to a single-strand RNA molecule. This RNA

transcript contains the same sequence of bases as the DNA strand except that uracil is substituted for thymine. The transcript is subsequently processed into the mature form known as the mRNA, in which the coding portions have been spliced together into a single continuous sequence, with the intervening sequences being discarded. The mRNA is modified in other ways to allow the subsequent translation of the information in the coding sequence into a protein. The level of expression of a gene is represented by the number of existing RNA transcripts that were produced from that gene within a given cell. The collection of RNA transcripts produced from all of the genes in the genome in a cell is known as the transcriptome, the study of which is known as transcriptomics. It is feasible to study the combined transcriptome of a set of cells, whether the cells reside in an organ or the cells are circulating in the bloodstream.

Of note, there are multiple mechanisms by which the process of transcription is regulated, allowing more or fewer RNA transcripts to be produced from a given gene. The region of DNA just upstream of the start of a gene, known as the promoter, can bind to a variety of proteins. Some of these proteins favor transcription, thus increasing the expression of the gene, whereas other proteins inhibit transcription, thus decreasing gene expression. Certain regions of DNA that are physically separate from the gene and can lie as far as megabases (millions of bases) away have the ability to bind proteins that can modulate transcription of a gene from a distance; these regions are known as enhancers and repressors. The proteins responsible for modulating transcription are known as transcription factors.

In addition to the protein-coding genes in the genome, several thousand regions can be transcribed into RNA molecules that do not contain information that encodes proteins but can nevertheless modulate the expression of protein-coding genes. Two well-described classes of so-called noncoding RNAs are microRNAs and long intervening noncoding RNAs. Through different mechanisms, both microRNAs and long intervening noncoding RNAs can affect the transcription of genes or the stability of their RNA transcripts, conferring an additional level of control of gene expression.

Within a chromosome, the DNA molecule is part of a complex known as chromatin, which includes a group of proteins known as histones. Depending on the configuration of these histones, which can be altered via biochemical modifications, an area of a chromosome may be more open or closed to transcription. Biochemical modifications of DNA bases within or near a gene sequence can also affect the level of transcription of the gene. The most common such modification is methylation of cytosine bases. In general, methylation tends to result in reduced transcription, thus silencing the gene. The collection of biochemical alterations to the DNA sequence and histone proteins within the chromosomes in the genome is known as the epigenome, and the study of such alterations is known as epigenomics.

The information encoded in mRNAs is used to produce proteins via the process of translation. Ribosomes scan through the coding sequence in an mRNA molecule and use each set of 3 bases, known as a codon, to specify a particular amino acid. The amino acids are attached in series, forming a lengthening protein strand. After a ribosome finishes scanning

the coding sequence, the completed protein is released from the ribosome and is then transported to the appropriate compartment of the cell or secreted into an extracellular space, for example, the bloodstream. Proteins can serve in a variety of roles, including as structural elements, as enzymes that catalyze biochemical reactions, as transcription factors, and as secreted hormones that foster communication between different types of cells. The collection of proteins present within a cell, within an organ, or within a fluid space (eg, bloodstream) is known as the proteome, and the study of the proteome is known as proteomics.

Enzymatic proteins act on a variety of substrates. In some cases, the substrates are other proteins, resulting in biochemically modified proteins with important functional consequences (an example being histone proteins in chromosomes). Other types of macromolecules also are amenable to enzymatic action. The products of enzymatic reactions and other biochemical processes are known as metabolites and can be present within cells or in extracellular compartments. The substrates that are converted to metabolites can originate from within the body or can be ingested. The collection of metabolites present within a cell, an organ, or a fluid space (eg, bloodstream) is known as the metabolome, the study of which is known as metabolomics.

Transcriptomics, epigenomics, proteomics, and metabolomics represent the analysis of macromolecule profiles that encompass different aspects of the expressed genome. These profiles are not simply a carbon copy of the information encoded in the genome (DNA) but reflect the interplay of the genetic information with a variety of environmental influences that impinge on the processes of transcription, translation, and enzymatic action. Just as variations in the DNA sequence can be studied with respect to their relationships with various phenotypes and diseases, so too can each class of macromolecules. It should be apparent that the functional consequences of genetic information that is combined with environmental influences are not limited to changes in these macromolecules but can manifest, and be measured, with respect to higher-order phenomena in the body. Whereas circulating cells in the bloodstream can be collected and studied with respect to the macromolecules carried within them, other properties of the cells such as the composition and number of cells in the compartment also are relevant to diseases. Important properties of noncirculating cells and tissues can be assessed through increasingly sophisticated, noninvasive molecular imaging techniques.

One important distinction between genetics and omics analyses is the temporality and dynamic changes observed in omics profiles. Genetic factors are fixed throughout the lifetime, so there is no need to be concerned about the temporality of effects and thus no issue of timing of sample collection. On the other hand, omics measures generally reflect a dynamic process, raising the issues of determining the optimal timing of measurements relative to disease onset and the possibility of reverse causation, that is, the disease driving changes in omics profiles rather than the omics profiles reflecting causation of the disease.

As related throughout the remainder of this document, profiling of each of these various aspects of the expressed

genome can potentially provide unique information that aids in the refinement of disease mechanisms and in the diagnosis and prediction of cardiovascular diseases and stroke.

General Considerations

In terms of the contribution of different categories of biomarkers from the expressed genome for diagnosis and prediction, some general considerations should be remembered. These include things such as clinical phenotyping (specifying the question and patient population), methodological readiness, refinement of mechanisms, and how to apply the knowledge in the clinical setting. In this section, we provide some general thoughts and guidelines on how to address these issues that are relevant for each of the subsequent sections that discuss different categories of biomarkers and methodologies.

Clinical Phenotyping

A phenotype refers to an observable trait. Technically speaking, even a genotype is a type of a phenotype because it is observable. In clinical use, phenotyping refers to the trait that is being studied such as myocardial infarction (MI) or stroke. A phenotype such as MI may have numerous subphenotypes such as hypertension, hyperlipidemia, or sensitivity to tobacco use, each of which may also have its own subphenotypes such as salt sensitivity, lipid plaque stability, or nicotine addiction. The harmonization of phenotypes refers to having a common definition of the phenotype being studied. For example, in the evaluation of hypertension, different studies may have defined hypertension differently. Such definitions include clinician diagnosis, actual blood pressure testing, cutoffs for systolic and diastolic pressures or either one alone, presence of hypertensive end-organ conditions such as left ventricular hypertrophy, or use of a medication to treat hypertension.

Although one may desire that all studies participating in a project use exactly the same definition of a phenotype to minimize heterogeneity, this often comes at an unnecessary sacrifice of statistical power. For example, one study might have diagnosed hypertension by actual blood pressure testing without treatment, and another study of a similar size might have used the same definition but also included patients with left ventricular hypertrophy if no other causes of the hypertrophy were present. The slight loss in phenotypic purity in the second study may be more than offset by the increased sample size (and consequent increase in power) to detect a relationship to hypertension. All of the subjects likely had hypertension even if the definitions differed slightly by study. When large consortia evaluate findings from multiple different studies, meta-analyses can evaluate the heterogeneity of the results and outliers in the results. If slightly different definitions were used but the heterogeneity is minimal across results from different studies, it is reassuring that the slight variation did not affect the overall results and interpretation. Alternatively, when high levels of heterogeneity occur, how one study differs from the next (such as regarding phenotypic definition) should be carefully considered. If available, information on the reliability and validity of a definition of a phenotype can be useful in determining which definition to use. Marginal increases in reliability and validity may not

ultimately be worth the sacrifice of power lost from using an overly parsimonious system. In general, as long as secondary analyses using a more refined definition in subgroup analyses can be performed, most studies have tended toward greater inclusiveness to increase power.

Controls are purest when they do not have the phenotype being studied; are similar or matched by age, race/ethnicity, sex, and geographic population; and are randomly selected from the population. Controls frequently do not undergo the same level of testing that cases do to ensure that they do not have the underlying condition or phenotype (if asymptomatic). However, if the frequency of the condition is expected to be low among the controls, the loss of power from requiring testing will offset the marginal misclassification bias of having a small portion of the controls with the phenotype of interest. Demographics and geographic population should be evaluated to avoid identifying relationships to those factors rather than to the phenotype of interest.

In summary, a phenotype is an observable trait. In practice, the necessity of high power to detect small effect sizes tends to have greater influence in deciding how to define a phenotype to permit greater inclusiveness, provided that the level of heterogeneity can be evaluated and, if necessary, accounted for.

Refinement of Mechanisms

An important consideration in the evaluation of novel biomarkers is whether they can assist in the refinement of mechanisms. Although at least in principle this is irrelevant for disease diagnosis, prediction, and prognosis, it is highly relevant for understanding the underlying pathophysiology of cardiovascular diseases; therefore, it is essential for the development of new therapeutic approaches. Hence, refinement of disease mechanisms is usually, and rightfully so, one of the main objectives of any study of biomarkers from the expressed genome. This increased understanding of disease mechanisms is often also the most important contribution of these biomarker studies, especially in early-stage discovery studies of novel biomarkers for cardiovascular diseases.

Biomarker studies can broadly be divided into targeted (or candidate-driven) and untargeted (or discovery) approaches. The targeted approaches are based on a hypothesis about a specific mechanism or pathway; therefore, they typically focus on 1 or a few biomarkers representing that biological system. In contrast, the untargeted approaches are hypothesis free, or rather hypothesis generating, and aim at surveying a whole class of biomarkers in an unbiased way. The use of untargeted approaches is a hallmark of omics studies. Many contemporary omics studies aim at assessing the whole genome, transcriptome, and increasingly the whole proteome or metabolome. In general, advantages of targeted approaches include lower burden of statistical testing (and hence less risk for false-positive findings), analytical assays that are specifically developed for the biomarkers in question, and less complex bioinformatics analyses. However, the main disadvantages of targeted approaches include being restricted to hypotheses from prior biological knowledge and the hypothesis testing being limited to the specific biological system being surveyed. In human genomics, unbiased genome-wide association studies dramatically changed the whole field, going from a handful

to thousands of robust genetic associations in just a few years. The learning experience was that untargeted approaches, if properly applied with adequate sample sizes and independent replication of top associations, can be very efficient in terms of finding new biomarkers that can help refine mechanisms and possibly change clinical care.²

Another aspect when considering refinement of mechanisms is the tissue specificity of a biomarker. The majority of biomarker studies, especially large-scale studies from the population, have been performed in a biological matrix easily accessible to the research team such as serum or urine. This may be adequate for some research questions and biomarkers, but it may be less suitable for others. For example, if the purpose of a project is to find circulating protein markers that can be easily measured in point-of-care testing to diagnose MI in patients with chest pain, a blood sample is clearly adequate. However, if the purpose is to refine tissue and cellular mechanisms involved in cerebral small vessel disease, blood or urine samples are less likely to be as useful. Therefore, it is always important to be aware of the research question and to consider the fidelity of the proxy tissue used if the primary tissue is not accessible.

Application of New Knowledge in the Clinical Setting

Although much of the content of this statement relates to new knowledge provided by omics studies into pathogenetic mechanisms of cardiovascular diseases, the ultimate objective of the search for this new knowledge is the improvement of patient care. Therefore, a critical consideration as to whether findings gleaned from new omics technologies are appropriate for real-world application to cardiovascular disease prediction and treatment is the need for independent external replication of the findings. There is still much work to be done with respect to most of the findings reported in this statement, precluding any serious consideration of their application in a clinical context at the present time.

This issue was addressed by an Institute of Medicine report published in 2012, *Evolution of Translational Omics: Lessons Learned and the Path Forward*,³ that was necessitated in part by the erroneous application in clinical practice of omics findings that lacked external validity, potentially causing harm to patients. This report described a 3-step process of translating omics to the clinic: the discovery phase, the test validation phase, and the evaluation for clinical utility and use phase.

Prompted by the Institute of Medicine report, the National Cancer Institute convened a working group of scientists representing multiple areas of expertise relevant to omics-based test development and other stakeholders, resulting in the publication of a checklist of 30 criteria for determining the readiness of omics-based tests to guide patient care in clinical trials (Table).⁴ The intent of the checklist is to evaluate proposals for National Cancer Institute–sponsored clinical trials in which omics tests are to be used. Although not all of the 30 specific criteria may be directly relevant to every conceivable application of omics findings, it can nevertheless be instructive to broadly consider the underlying principles in judging whether an application has been appropriately evaluated for clinical use. In the next 2 sections, we describe some of the

considerations highlighted by the Institute of Medicine and National Cancer Institute publications.

Methodological Readiness

One of the key questions in the evaluation of novel biomarkers concerns the analytical methods and their readiness for scaling, point-of-care measurements, and clinical implementation. Hence, in the evaluation of a novel biomarker, it is important to understand how the assay was devised and validated. First, what were the circumstances under which the novel biomarkers were discovered? What were the characteristics of the study population? In what conditions were specimens obtained? What analytical method was used for the biomarker measurement? How were the diagnostic classifiers developed and evaluated? Second, how was the assay externally validated? Was the optimal timing for the measurement of analytes assessed? What were the clinical characteristics of the patient populations? What were the details of the statistical analysis plans, and were they developed before or after the analysis was performed? All of these questions, as well as other questions about the assay and analytical methods, are relevant in the interpretation of results of a research study reporting a novel promising biomarker for the diagnosis or prediction of a cardiovascular disease.

As a first step, the readiness of a novel biomarker assay for translation to clinical practice can be evaluated by assessment of the quality of the underlying science. For example, a biomarker might have been discovered in a large study population that is a good representation of the underlying population in which the biomarker should be used. A second biomarker might have been discovered in a small study population with many inclusion and exclusion criteria. The first biomarker is more likely to be ready for clinical translation than a biomarker discovered in a small study population with many inclusion and exclusion criteria. Similarly, an assay that has already been implemented at a core hospital laboratory is readier for translation than an assay developed for the specific study using dedicated, specific methods that can be difficult for other laboratories to replicate. Furthermore, the diagnostic classifiers used should be reliable, validated, and therapeutically relevant rather than developed specifically for that biomarker study. Finally, there should be evidence that the novel biomarker provides incremental information when added to well-established clinical risk factors and biomarkers.

One common obstacle when trying to go from the discovery of novel biomarkers to clinical implementation is that the analytical methods and regulatory requirements are very different in the phases of scientific discovery and clinical application. For example, the discovery of novel biomarkers can be done with any method, laboratory, and personnel as long as ethical permits are adhered to and the peer review process recognizes the science as being sound. In contrast, the clinical application of a biomarker assay involves certifications and quality standards set forth by regulatory authorities such as the US Food and Drug Administration and Centers for Medicare & Medicaid Services through the Clinical Laboratory Improvement Amendments.⁵ Apart from challenges arising from regulatory issues, there are often technical barriers to the readiness for scaling, point-of-care measurements, and

Table. National Cancer Institute Criteria for the Use of Omics-Based Predictors in Clinical Trials

Domain	Criteria
Specimen issues	<p>Establish methods for specimen collection and processing and appropriate storage conditions to ensure the suitability of specimens for use with the omics test.</p> <p>Establish criteria for screening out inadequate or poor-quality specimens or analytes isolated from those specimens before performing assays.</p> <p>Specify the minimum amount of specimen required.</p> <p>Determine the feasibility of obtaining specimens that will yield the quantity and quality of isolated cells or analytes needed for successful assay performance in clinical settings.</p>
Assay issues	<p>Review all available information about the SOPs used by the laboratories that performed the omics assays in the developmental studies, including information on technical protocol, reagents, analytical platform, assay scoring, and reporting method, to evaluate the comparability of the current assay to earlier versions and to establish the point at which all aspects of the omics test were definitively locked down for final validation.</p> <p>Establish a detailed SOP to conduct the assay, including technical protocol, instrumentation, reagents, scoring and reporting methods, calibrators and analytical standards, and controls.</p> <p>Establish acceptability criteria for the quality of assay batches and for results from individual specimens.</p> <p>Validate assay performance by using established analytical metrics such as accuracy, precision, coefficient of variation, sensitivity, specificity, linear range, limit of detection, and limit of quantification, as applicable.</p> <p>Establish acceptable reproducibility among technicians and participating laboratories and develop a quality assurance plan to ensure adherence to a detailed SOP and to maintain reproducibility of test results during the clinical trial.</p> <p>Establish a turnaround time for test results that is within acceptable limits for use in real-time clinical settings.</p>
Model development, specification, and preliminary performance evaluation	<p>Evaluate data used in developing and validating the predictor model to check for accuracy, completeness, and outliers. Perform retrospective verification of the data quality if necessary.</p> <p>Assess the developmental data sets for technical artifacts (eg, effects of assay batch, specimen handling, assay instrument or platform, reagent, or operator), focusing particular attention on whether any artifacts could potentially influence the observed association between the omics profiles and clinical outcomes.</p> <p>Evaluate the appropriateness of the statistical methods used to build the predictor model and to assess its performance.</p> <p>Establish that the predictor algorithm, including all data preprocessing steps, cut points applied to continuous variables (if any), and methods for assigning confidence measures for predictions, are completely locked down (ie, fully specified) and identical to prior versions for which performance claims were made.</p> <p>Document sources of variation that affect the reproducibility of the final predictions and provide an estimate of the overall variability along with verification that the prediction algorithm can be applied to 1 case at a time.</p> <p>Summarize the expected distribution of predictions in the patient population to which the predictor will be applied, including the distribution of any confidence metrics associated with the predictions.</p> <p>Review any studies reporting evaluations of the performance of the predictor to determine their relevance for the setting in which the predictor is being proposed for clinical use.</p> <p>Evaluate whether clinical validations of the predictor were analytically and statistically rigorous and unequivocally blinded.</p> <p>Search public sources, including literature and citation databases, journal correspondence, and retraction notices, to determine whether any questions have been raised about the data or methods used to develop the predictor or to assess its performance and ensure that all questions have been adequately addressed.</p>
Clinical trial design	<p>Provide a clear statement of the target patient population and intended clinical use of the predictor and ensure that the expected clinical benefit is sufficiently large to support its clinical utility.</p> <p>Determine whether the clinical utility of the omics test can be evaluated with stored specimens from a completed clinical trial (ie, a prospective-retrospective study).</p> <p>If a new prospective clinical trial will be required, evaluate which aspects of the proposed predictor have undergone sufficiently rigorous validation to allow treatment decisions to be influenced by predictor results; when treatment assignments are randomized, provide justification for equipoise.</p> <p>Develop a clinical trial protocol that contains clearly stated objectives and methods and an analysis plan that includes justification of sample size; lock down and fully document all aspects of the omics test and establish analytical validation of the predictor.</p> <p>Establish a secure clinical database so that links among clinical data, omics data, and predictor results remain appropriately blinded, under the control of the study statistician.</p> <p>Include in the protocol the names of the primary individuals who are responsible for each aspect of the study.</p>
Ethical, legal, and regulatory issues	<p>Establish communication with the individuals, offices, and agencies that will oversee the ethical, legal, and regulatory issues relevant to the conduct of the trial.</p> <p>Ensure that the informed consent documents to be signed by study participants accurately describe the risks and potential benefits associated with use of the omics test and include provisions for banking of specimens, particularly to allow "bridging studies" to validate new or improved assays.</p> <p>Address any intellectual property issues concerning the use of the specimens, biomarkers, assays, and computer software used to calculate the predictor.</p> <p>Ensure that the omics test is performed in a Clinical Laboratory Improvement Amendments–certified laboratory if the results will be used to determine treatment or will be reported to the patient or the patient's physician at any time, even after the trial has ended or the patient is no longer participating in the study.</p> <p>Ensure that appropriate regulatory approvals have been obtained for investigational use of the omics test. If a prospective trial is planned in which the test will guide treatment, consider a presubmission consultation with the US Food and Drug Administration.</p>

SOP indicates standard operating procedure.

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clinical implementation of novel biomarker assays. Large development costs and efforts may be involved in the translation of an assay from a small-scale, in-house measurement to an assay that can be performed at a larger scale with the high quality needed for clinical implementation.

Utility of New Tests in the Clinical Setting

Ultimately, the overall purpose of any biomedical research is that it should improve human health, often by advancing clinical care. There are several ways that increased knowledge about the expressed genome can achieve this. Knowledge of the underlying pathophysiology of diseases is the first step toward new therapeutic approaches, as discussed above. The road from an initial observation in an omics study to an approved therapy that can be used to treat patients is long and complicated, but drug development is nevertheless one of the key rationales for any studies of the expressed genome. Another common goal for these studies, which at least in theory could lead to clinical applications more swiftly, is the development of biomarker tests to be used to predict disease risk, to diagnose disease, and to prognosticate risk of recurrence or other adverse outcomes.

The methods used to study novel biomarkers in prediction, diagnosis, and prognosis, as well as previously nominated biomarkers, have been thoroughly described and evaluated elsewhere.^{6–8} Briefly, they involve measures of association, discrimination, calibration, reclassification, and cost-effectiveness. Other important aspects in the evaluation of biomarkers include safety, replication across different populations, needs to re-educate clinicians and the general public, and usefulness for motivating patients. The first requirement of a novel biomarker is that it is robustly associated with the outcome in question, preferably across different study populations and after established predictors are taken into account. However, when clinical usefulness is being judged, a significant *P* value from a multivariable model is far from enough. Adequate assessment of biomarkers for prediction, diagnosis, or prognosis typically also should involve metrics of discrimination, calibration, and reclassification.

Discrimination refers to the capacity of a test to separate individuals who go on to develop disease (prediction), who are diseased (diagnosis), or who go on to have an adverse event (prognosis) from individuals who do not. The most common discrimination metric is the C statistic or the area under the receiver-operating characteristic curve, which combines the sensitivity (true-positive rate) and specificity (true-negative rate) of a test.⁹ This metric reflects the probability that a randomly selected individual from the diseased group has a higher predicted risk than a randomly selected individual from the nondiseased group. Hence, a C statistic of 1.0 means a perfect test, whereas a C statistic of 0.5 means a worthless test providing no information beyond randomness. For the prediction of coronary heart disease in a previously healthy population, a model including only age and sex typically yields a C statistic of 0.65 to 0.70, whereas the Framingham Risk Score reaches ≈ 0.75 .¹⁰

Calibration reflects the agreement between predicted and observed risk across groups of individuals with different baseline risk. A commonly used metric is the Hosmer-Lemeshow

statistic,¹¹ although graphical models (calibration curves) are commonly used. An intrinsic limitation of both discrimination and calibration metrics is that they give equal importance to individuals across the whole risk spectrum, which may not be the case in the real clinical setting. In practice, biomarkers may be most useful when applied in a specific, narrower, range of risk as a “tiebreaker test” in individuals with intermediate risk for whom additional knowledge really can make a difference in the clinical decision making.

Reclassification metrics have been introduced to address this need, and the measure that has become most popular is the net reclassification index.¹² The net reclassification index gives an assessment of the extent to which the addition of a novel biomarker to a risk prediction model moves (or reclassifies) individuals across a predefined risk threshold. This metric is most suitable in situations in which there are established risk thresholds at which the clinical management changes. One example is the 5% and 7.5% absolute 10-year risk thresholds for atherosclerotic cardiovascular disease in the 2013 American College of Cardiology/American Heart Association guidelines on lipid management.¹³ The net reclassification index is also useful in the sense that it translates the continuous nature of a risk gradient arising from a risk prediction equation into a clinically actionable yes-or-no decision; that is, to treat or not to treat. In addition to the most common metrics, discrimination, calibration, and reclassification, different measures addressing cost-effectiveness can be useful for evaluating novel biomarkers and their utility in the clinical setting. For example, the number needed to test reflects the number of individuals who need to be tested to correctly predict, diagnose, or prognosticate an additional event. This can be a useful metric, especially when balanced against the cost, patient burden, and side effects of the test in a cost-effectiveness analysis.

Some existing risk algorithms, for example, the prediction of coronary heart disease in healthy populations, are already quite accurate, and as a result, additional risk markers need to show very large effect sizes to show any improvement in C statistics.¹⁴ It has been argued that it is a waste of time and resources to perform additional studies trying to discover novel biomarkers for risk prediction and that it would be better to focus on applying preventive measures known to be effective. This point certainly has some validity, but it is important to recognize a few caveats. Population-based primary prevention strategies have had very limited success in the past (for a number of reasons). A combination of a larger number of novel biomarkers such as those arising from omics studies of the expressed genome is more likely to show substantial improvements in risk prediction (and there is still much room for improvement, even in the situations in which the risk algorithms work relatively well). There are many clinical situations in which there is a lack of good prediction algorithms. Finally, as pointed out above, risk prediction is just one potential use of studies of the expressed genome. In many cases, the primary goal of these studies is to learn more about biology and disease pathophysiology, which in turn can lead to new treatment paradigms.

In summary, the development of novel biomarker tests for diagnosis, risk prediction, and prognosis is an exciting

application of studies of the expressed genome. There are well-established methods for evaluating such novel biomarkers that can and should be applied. This is true regardless of whether the biomarker test is a point-of-care test used in the acute setting or whether it is a test that is used in the primary care setting or even sent to the patient's home to be used in long-term, preventive care. These prediction methods are often underused or misused,⁷ and increased educational efforts addressing the application of these methods would benefit the omics field as a whole. In future studies, it will be important to consider the clinical question already in the study design rather than to get distracted by fancy high-throughput methods, to think about how a novel biomarker can add to the existing clinical paradigm, and to emphasize unmet needs, for example, in intermediate-risk populations in whom a biomarker can function as a tiebreaker test.

Transcriptomics

The generation of RNA from DNA is the first step in the transition from the inherited to the expressed genome. Quantifying the levels of RNA molecules for all or a subset of the $\approx 20\,000$ coding genes in the genome, as well as microRNAs and long intervening noncoding RNAs, is referred to as transcriptomics or gene expression profiling. For analysis of a limited number of gene transcripts, some version of the polymerase chain reaction (PCR) is frequently used such as real-time or quantitative PCR. For increased sensitivity to detect rare transcripts, "digital" PCR and single-molecule imaging techniques have emerged. For analysis of a larger number of transcripts such as all known transcripts in the genome, microarray technology quickly became the platform of choice. Microarrays use DNA oligomers that are immobilized onto a glass slide; the oligomers are chosen to specifically match sequences in the desired genes. The expression level of a gene in a sample is quantified by measuring the amount of RNA that binds to a matching oligomer through Watson-Crick base pairing. Just as next-generation sequencing has revolutionized the analysis of the inherited genome, next-generation sequencing has recently replaced microarrays as the preferred platform for gene expression analysis by allowing an unbiased approach to transcript discovery and quantification at a comparable cost.

Although the expressed genome ultimately results from multiple subsequent processes after the transcription of DNA into RNA such as translation, variation in the levels of RNA (specifically mRNA) transcripts can explain a large proportion of the variation in protein levels.¹⁵ Because of the dynamic nature of transcriptional regulation, RNA levels represent not only features encoded in the genome (described above) but also the influence of the environment (eg, air pollution, diet, medications). Because genes are often transcribed in a coordinated fashion, one can infer pathways that are associated with a phenotype of interest by finding concordance of statistical associations across a large number of genes. Coordinated gene expression often leads to a statistical advantage in bioinformatics analyses because the expression levels of dozens to hundreds of genes are correlated with each other, therefore reducing the dimensionality (ie, complexity) of a gene expression data set.¹⁶ This feature, coupled with the fact that only a fraction of the $\approx 20\,000$ genes in the genome are expressed in a

given tissue, further narrows the statistical haystack in which to look for associations. Finally, the ability to measure RNA levels in a commercial diagnostic testing laboratory has been well established. For example, measuring RNA levels of hepatitis C virus or HIV viral loads has been standard diagnostic testing available to physicians for decades.

Thus, a multitude of reasons have motivated investigators to use gene expression profiling as the starting point for biomarker discovery and identification of novel disease mechanisms in the cardiovascular system. The focus of this section is the analysis of mRNAs derived from blood and myocardium in the development, diagnosis, prognosis, and treatment of CAD and its sequelae.

It is well known that CAD is often heralded by traditional risk factors such as smoking, diabetes mellitus, blood lipid levels, and hypertension. However, what is less well understood is how these risk factors lead to CAD. By studying associations with specific risk factors, investigators aim to highlight novel mechanisms underlying CAD. The rationale for these studies, typically performed with blood, is that risk factors create a proatherosclerotic milieu that is captured in the RNA profiles of circulating cells. For example, a series of studies using microarray analysis of either unfractionated peripheral blood or purified lymphocytes across the spectrum of tobacco smoke exposure compared with never smokers have identified several genes representing inflammatory pathways that may contribute to CAD.^{17–19} Those with secondhand smoke exposure could not be distinguished from primary smokers,¹⁷ and neither could recent smokers (quit <2 months previously) be separated from current smokers.¹⁹ However, former smokers (quit >2 months previously) most closely resembled never smokers from the perspective of their gene expression in peripheral blood.¹⁹ These relationships suggest a dose-response relationship, reinforce the dangers of secondhand smoke exposure, and confirm that smoking cessation is associated with reversible effects on biological pathways that may underlie CAD.

Diabetes mellitus is a powerful magnifier of CAD risk. Analysis of hepatic and circulating peripheral blood mononuclear cells with microarray techniques identified the c-Jun N-terminal kinase and oxidative phosphorylation pathways as being dysregulated in patients with type 2 diabetes mellitus.^{20,21} As further evidence that hyperglycemia drives these changes, when patients with diabetes mellitus were evaluated before and after achieving glycemic control, there was normalization toward the nondiabetic state in c-Jun N-terminal kinase pathway genes, reinforcing the potential benefits of euglycemia in patients with diabetes mellitus.²⁰ In terms of less conventional CAD risk factors, body mass index and the metabolic syndrome are associated with the inflammatory nuclear factor- κ B pathway²² and innate immune response,²³ respectively. Thus, through gene expression analysis of risk factors for CAD, several pathways have emerged related to inflammation, a well-known driver of atherosclerosis.²⁴

In contrast to investigations focused on risk factors, others have sought to directly study CAD in an attempt to identify novel mechanisms and diagnostic biomarkers. Initial attempts compared patients with CAD with healthy control subjects without independent validation of their findings,

thus limiting their conclusions.^{25,26} The largest body of work around gene expression profiling for CAD is related to the development of a clinically available diagnostic test for CAD (Corus CAD). Because the focus of these studies was the development of a novel diagnostic test, the choice of patient populations, study design, and RNA transcripts was driven by the need to build a predictive model for a specific diagnostic test rather disease pathobiology. Nevertheless, the culmination of this work with microarray analysis led to the discovery of genes associated with the presence and extent of CAD.²⁷ The final algorithm uses age, sex, and the expression levels of 23 genes in peripheral blood RNA as assessed by PCR to classify obstructive CAD (ie, $\geq 50\%$ stenosis in at least 1 major coronary artery).²⁸ The diagnostic accuracy of the algorithm was demonstrated in prospective independent cohorts with a sensitivity of $\approx 85\%$ and a negative predictive value of $\approx 85\%$ in diagnosing CAD.^{29,30} Although the algorithm applies only to patients without diabetes mellitus and, unlike traditional stress testing, does not provide prognostic information, it provides a validated noninvasive laboratory test to diagnose obstructive CAD.

Most gene expression studies of CAD have focused on patients with stable CAD. However, patients with acute coronary syndromes such as MI likely exhibit unique biological processes that drive the transition from stable to unstable CAD. Platelets are well known to contribute to the development of MI and are also known to contain a large amount of functional RNA,^{31–33} despite lacking nuclei. Among patients presenting with MI, platelet gene expression analyses have identified genes and pathways linked to the adaptive immune response.^{34,35} Similar studies using peripheral blood mononuclear cells have also identified genes related to adaptive immunity as being associated with MI.³⁶ Of note, most studies of the expressed genome in MI cannot determine whether changes in gene expression are the cause or the result of MI. However, platelets lack nuclei, and their mRNA levels are established at the time of megakaryopoiesis. Thus, it may be that pathological changes in platelet RNA can precede MI by at least several weeks, allowing the possibility of monitoring patients at risk of near-future MI.

Antiplatelet therapy with aspirin is a cornerstone of the treatment and prevention of MI. Variability in platelet response to aspirin has been well described and includes mechanisms beyond the inability of aspirin to inhibit platelet cyclooxygenase-1. Peripheral blood gene expression profiling has identified a 60-gene signature that is of platelet origin and both correlated with platelet function in response to aspirin and associated with cardiovascular events in patients with CAD treated with aspirin.³⁷ In a unique approach, investigators have used lymphoblastic cell lines from patients given simvastatin for ex vivo gene expression profiling of lymphoblastic cell lines before and after exposure to simvastatin. The goal of these experiments was to link genetic variation, gene expression profiles from ex vivo drug exposure, and in vivo drug responses to identify novel mechanisms of simvastatin response. These ex vivo experiments have uncovered novel pathways that underlie variation in low-density lipoprotein cholesterol lowering³⁸ and novel genetic variants associated with musculoskeletal side effects in response to simvastatin.³⁹

Gene expression profiles for other commonly used cardiovascular medications have not yet been developed. However, several medications (eg, clopidogrel, metoprolol, warfarin) are metabolized by cytochrome P450 enzymes that are known to be highly variable in activity among individuals. Hepatic gene expression profiling correlates with cytochrome P450 activity, and one study has linked peripheral blood cytochrome P450 gene expression with hepatic cytochrome P450 activity,⁴⁰ thus laying the foundation for a novel set of biomarkers to inform drug dosing and selection.

The progression from MI to systolic heart failure is variable and is driven by factors related to the underlying event (ie, infarct size, timeliness of reperfusion, and concomitant medical therapy), as well as complex biological processes (ie, inflammation, regulation of extracellular matrix turnover, fibrosis, cell death, and angiogenesis). To better understand these processes, peripheral blood gene expression analysis with microarrays at the time of acute events was used to discover and validate *TGFBR1* as a promising candidate.⁴¹ Expression of *TGFBR1* in peripheral blood could predict a reduced ejection fraction after MI independently of markers of infarct size (eg, troponin T and creatine kinase). *TGFBR1* expression in this study was likely measured from circulating monocytes/macrophages, and the transforming growth factor- β pathway is well known to influence the switch from an inflammatory response to a fibrotic response after MI.⁴²

Ischemic cardiomyopathy is a common consequence of CAD and MI and can lead to sudden cardiac death (SCD) caused by ventricular arrhythmias. A reduced ejection fraction is a well-known risk factor for SCD and is the key criterion for primary prevention with implantable cardioverter-defibrillator (ICD) therapy. However, the majority of patients who receive an ICD never need it, and many more who do not qualify for an ICD go on to experience SCD.⁴³ There is therefore a need for improved tools to more precisely identify patients with ischemic cardiomyopathy who are at risk for SCD. One approach has been to study the underlying electrophysiological properties of the myocardium in heart failure. The *SCN5A* gene encodes the primary sodium channel in myocardium. Well-described *SCN5A* genetic variants that reduce the function of this channel and thus reduce the sodium current lead to an increased risk for SCD in Brugada syndrome.⁴⁴ Microarray analysis of normal versus failing human myocardium identified upregulation in specific splicing factors in heart failure that led to increased levels of a truncated, nonfunctional version of *SCN5A* that reduces sodium current.⁴⁵ Interestingly, aberrant *SCN5A* splicing is also observed in peripheral blood cells from patients with heart failure and is tightly correlated with myocardial *SCN5A* mRNA levels, thus providing a peripheral blood “window” into the electric properties of the heart.⁴⁶ Peripheral blood *SCN5A* expression profiling with PCR predicted appropriate ICD shock therapy in patients with heart failure with high accuracy.⁴⁶ These early data lay the foundation for a novel gene expression–based biomarker to help risk-stratify patients for ICD therapy.

A small minority of patients with ischemic cardiomyopathy fail best medical therapy and progress to end-stage heart failure requiring left ventricular assist device therapy. A subset of patients who receive left ventricular assist device

therapy recover left ventricular function (reverse remodeling) to the point where the left ventricular assist device can be explanted.⁴⁷ This process has provided a unique opportunity to access myocardial tissue for gene expression analysis before and after reverse remodeling to identify mechanisms that are associated with improvement in left ventricular function with mechanical unloading. The reverse remodeling process appears to be associated with genes in the integrin pathway,⁴⁸ arginine metabolism,⁴⁹ and Wnt signaling,⁵⁰ whereas failure to recover function was associated with profibrotic genes such as *TGFBI*.⁵¹

For patients with end-stage heart failure for whom left ventricular assist device support is not indicated or required, cardiac transplantation is sometimes an option. Transplantation significantly improves heart failure symptoms but adds the risks of rejection (acute and chronic), immunosuppressive therapy, and opportunistic infections. The gold standard for surveillance of cardiac allograft rejection is an invasive, endocardial biopsy. From myocardial biopsies and peripheral blood for microarray analysis, diagnostic signatures for acute rejection^{52,53} and chronic rejection⁵⁴ have been identified. The latter has been developed into a clinically available diagnostic test (AlloMap) using RNA from peripheral blood, PCR, and a validated algorithm that identifies patients with chronic rejection. Compared with a strategy of routine biopsies, use of the AlloMap test was noninferior with similar rejection, graft dysfunction, death, and retransplantation rates, similar adverse outcomes, and fewer biopsies.⁵⁵

At every step in the natural history of CAD, from risk factors to diagnosis in symptomatic patients to many of the sequelae, including MI, heart failure, arrhythmia, and transplantation, gene expression profiling of blood and myocardial tissue has identified novel biological processes underlying these disorders and yielded novel diagnostic tests. Although informative, gene expression profiling is just one entry point into a continuum of experiments also involving genetics, proteomics, metabolomics, etc, to further refine disease mechanisms. Peripheral blood gene expression profiling lends itself to diagnostic testing such as Corus CAD and AlloMap but is far removed from the tissues of interest in CAD such as endothelium, vasculature, and myocardium. Therefore, there are inherent limitations in blood-based signatures of diseases, and direct tissue profiling will likely be required. Finally, although the vast majority of transcriptomics studies have focused on mRNAs, a suite of noncoding RNAs expressed in blood and other key tissues contribute to disease and remain to be characterized. In particular, unbiased analyses of microRNA profiles in CAD and other cardiovascular diseases are now underway, although to date microRNA studies have been largely limited to the investigation of the relationships of individual microRNAs with disease, as reviewed elsewhere.^{56,57}

As described in previous sections, translating transcriptomic discoveries from research settings into the clinical environment is a nontrivial task that goes beyond independent validation. Successful translation additionally involves technical factors such as sample source, handling, and processing; statistical issues concerning the transparency of model development and validation; prospective clinical trial issues concerning clinical utility and performance; and regulatory issues

around Clinical Laboratory Improvement Amendments/the US Food and Drug Administration. The National Cancer Institute's set of 30 criteria to evaluate potential omics-based predictors for clinical use (Table) should be considered in judging whether a particular test is appropriate for patient care. The 2 transcriptomic tests discussed in this section, Corus CAD and AlloMap, have met many of these criteria.

The Corus CAD test was based on initial discovery using microarrays and independent validation using reverse-transcriptase PCR of genes that were identified for their collective ability to identify patients with or without obstructive CAD.²⁷ This initial discovery work led to the development of pre-defined technical and quality control parameters required for a diagnostic test using peripheral blood reverse-transcriptase PCR⁵⁸ and a statistical model using age, sex, and the expression of these 23 genes to identify nondiabetic patients with a high likelihood of obstructive CAD.²⁸ The performance of this retrospectively developed model was prospectively assessed in 2 studies^{29,30} in symptomatic patients with suspected CAD. The clinical utility of the Corus CAD test has also been prospectively assessed⁵⁹⁻⁶² with respect to the initial diagnostic workup of patients with suspected CAD. As a result of this work, the Corus CAD test is a clinically available diagnostic test that has been evaluated, has been deemed to be valid and useful, and accordingly is covered by many insurers. The AlloMap test has similarly been rigorously assessed in terms of developing and validating a statistical model with peripheral blood gene expression used to diagnose cardiac rejection.⁵⁴ The use of AlloMap in the management of cardiac rejection compared with routine endomyocardial biopsy was evaluated and validated in a prospective randomized clinical trial.⁵⁵ As a result, the US Food and Drug Administration has approved AlloMap as a diagnostic test to aid in the detection of chronic cardiac graft rejection.

Epigenomics

Broadly speaking, epigenetics is the study of external factors besides DNA sequence variation that influence the process of gene transcription. In principle, this can encompass a diverse set of mechanisms ranging from DNA methylation to histone modifications, transcription factors, microRNAs, and long intervening noncoding RNAs. The emergence of straightforward techniques for genome-scale analysis of DNA methylation and, to a lesser extent, histone modifications has resulted in the discipline of epigenomics, that is, the study of the full collection of these 2 types of biochemical alterations to the genome. Most of the disease-oriented efforts to date have focused on DNA methylation. These alterations can be dynamic in response to environmental influences during one's lifetime rather than solely being inherited from one's parents, as is typically the case with DNA sequence variation. Thus, epigenomics offers an important window through which one can observe how the expressed genome influences disease pathophysiology. However, unlike genetic variation, which is static and impervious to reverse causation, when epigenetic changes are observed to be associated with disease, it is difficult to know whether those particular changes are driving the disease process or the changes are a secondary consequence of

the disease process. In either case, knowledge of the epigenetic changes may have value if they provide biomarkers with which to predict the incidence or severity of the disease.

As a result of technical limitations, early studies focused on discerning whether there were associations between non-specific, global changes in methylation across the genome and CAD.⁶³ One study found that genomic DNA in peripheral blood cells from patients with CAD was generally hypermethylated compared with control individuals.⁶⁴ In contrast, another study focused on methylation of noncoding, repetitive long interspersed nucleotide element-1 elements, which occur with high frequency throughout the genome and are normally highly methylated. This study found that hypomethylation, not hypermethylation, of these elements in genomic DNA in peripheral blood cells was associated with prevalent CAD and stroke, incident CAD and stroke, and increased CAD and stroke mortality.⁶⁵ However, another study focused on methylation of noncoding Alu and satellite 2 repetitive elements throughout the genome and found that men, but not women, with CAD, hypertension, or diabetes mellitus displayed increased Alu and satellite 2 methylation in peripheral blood cells.⁶⁶ Of note, these 3 studies were performed in 3 different ethnic populations.

The emergence of methods to interrogate specific sites in the genome for methylation status has now made it possible to assess candidate genes and to perform unbiased epigenome-wide association studies, in analogy to genome-wide association studies performed with DNA variants. For example, an epigenomic array that analyzed methylation at 27 578 sites in >14 000 gene promoter regions in genomic DNA in peripheral blood cells identified a locus in the *F2RL3* gene for which hypomethylation was strongly associated with tobacco smoking.⁶⁷ Once identified, the *F2RL3* locus served as the basis of candidate gene studies that found that hypomethylation of the locus was strongly associated with mortality among patients with stable CAD⁶⁸ and was associated with cardiovascular mortality in a population cohort, particularly among men.⁶⁹ In another example, an epigenome-wide association study performed in peripheral blood cells from patients with familial hypercholesterolemia identified the *TNNT1* gene locus as a site at which decreased methylation was associated with decreased high-density lipoprotein cholesterol levels.⁷⁰ A follow-up candidate gene study with *TNNT1* found that in individuals without familial hypercholesterolemia, increased methylation was associated with CAD.⁷¹ In other candidate gene studies, increased methylation at *CDKN2B* was associated with CAD,⁷² increased methylation at *PLA2G7* was associated with CAD among women,⁷³ and increased methylation at *INS* and *GNASAS*, loci with a methylation status thought to be influenced by the prenatal environment, was associated with increased incidence of MI among women.⁷⁴ Although all of these findings are intriguing, for the most part, they have emerged from small sample sizes and need to be replicated in independent cohorts to establish their validity.

There has been a dearth of epigenome-wide association studies performed directly for CAD, as opposed to CAD risk factors, but one such study identified a number of candidate hypermethylated loci in peripheral blood cells from patients with CAD that await replication.⁷⁵ A greater number

of epigenome-wide association studies have been performed directly for cardiomyopathy using cardiac tissues from patients with end-stage disease and healthy individuals. Among the gene loci that have been reported to have altered methylation patterns in advanced heart failure are *DUX4*, *LY75*, *ERBB3*, *HOXB13*, and *ADORA2A*.^{76,77} The *DUX4* locus is particularly notable because it was also found to harbor changes in histone modifications,⁷⁶ a different type of epigenetic change that has not been well studied in cardiovascular disorders to date.

There is great interest in expanding the role of epigenomics to better understand the role of epigenetic changes in disease pathophysiology. Another incentive is to discover better disease biomarkers that can be used to more accurately predict disease risk, morbidity, and mortality and ultimately to guide treatment in individuals. To these ends, a number of international collaborative projects have been undertaken, including the Human Epigenome Project and the International Human Epigenome Consortium. These collaborations have worked to develop comprehensive catalogs of epigenetic markers within the human genome in a large variety of tissue types.

Proteomics

Proteomics refers to the determination of the protein complement of a tissue, cell, or fluid compartment such as serum or urine. The term proteome was proposed almost 2 decades ago,⁷⁸ at a time when the primary technologies used to profile the protein complement were considerably less advanced. Analysis was often limited to gel separation in 1 or 2 dimensions, followed by identifying proteins by gel extraction, determining partial amino acid composition of individual proteins, and matching them to nucleotide sequence databases. The field has advanced considerably in the past 2 decades by the application of newer technologies and applications of protein separation and mass spectrometry (MS)-based techniques to separate complex protein mixtures; to characterize proteins in greater depth, including a variety of posttranslational modifications; to use MS technology with greater sensitivity to detect low-abundance peptides; to develop new methods to accurately quantify proteins in a complex mixture and differences in abundance between sample sets; and to develop new bioinformatics tools to accurately categorize and compare highly complex data sets. Even more recently, antibody-based techniques are being applied to proteomics, potentially improving on the sensitivity of MS-based techniques.⁷⁹ It is notable that the use of serum protein biomarkers of cardiovascular diseases in a directed way preceded the development of newer technologies. Nevertheless, the advances made in proteomics technologies and strategies are poised to directly affect the study of cardiovascular diseases and stroke, as outlined in a recent American Heart Association scientific statement.⁸⁰ This section briefly discusses current protein biomarkers that were developed primarily in a hypothesis-directed fashion, as well as newer discovery-based approaches that may be transformative by identifying novel biomarkers in an unbiased way.

There are many challenges in the application of proteomics to advance our understanding of cardiovascular diseases. In cancer proteomics, diseased tissue may be directly available via its surgical removal at diagnosis. In contrast, availability of myocardial tissue or peripheral or cerebral

vascular tissue is often limited to retrieval either postmortem or in end-stage disease via myocardial biopsy, placement of mechanical cardiac support, heart transplantation, or other surgeries. Normal human cardiovascular tissue, which can serve as the baseline for comparison, also has limited availability. Therefore, most proteomics work to develop biomarkers in human cardiovascular diseases is limited to the blood compartment, which includes serum, plasma, and circulating cells. Although challenging, when proteomics is combined with increasingly sophisticated imaging strategies, there is the opportunity to correlate cardiovascular pathology with an altered proteome in blood.

Established cohorts such as the Framingham Heart Study, the Jackson Heart Study, and the Multi-Ethnic Study of Atherosclerosis, as well as numerous clinical trial tissue repositories, also offer opportunities for correlations with outcomes. Ideally, newly developed potential biomarkers must be determined from carefully collected and stored biospecimens and validated before correlation with disease states. Some of the challenges of this workflow have been noted in recent consortium and meeting statements.^{81,82} Biomarkers should be validated in >1 well-characterized sample set before application in a large-scale study. An important facet of assay development is attention to the use of appropriate control samples.

Characterization of protein biomarkers of cardiac injury in ischemic disease began well before the development of the field of proteomics and has transformed clinical cardiology over the past few decades. The history of the development of specific assays, including the creatine kinase assay and troponin assays, has been described by Jack Ladenson,⁸³ the clinical chemist who developed these assays. The approach used classic hypothesis-directed studies, with the understanding that specificity for myocardial tissue and higher sensitivity were desirable. The focus on troponin I and T assays was driven by recognition of their myocardial specificity. Troponin assays informed the definition of MI, and their improved sensitivity has led to use of these assays to detect possible myocarditis and heart failure. It is important to note that troponin levels may also be elevated in the blood of patients with a wide variety of other medical disorders.⁸⁴ Although these elevations reflect myocardial damage that is associated with poorer prognosis, the underlying mechanism for myonecrosis is not believed to be ischemic and varies by illness. It has also been recognized that even when MI has been excluded among patients who present to the emergency room with chest pain, low levels of troponin in blood, as detected by high-sensitivity assays, predict higher all-cause mortality and cardiac hospitalization in the months ahead.⁸⁴ Although immunoassays are the method used in current troponin assays, extension to even more sensitive MS technologies may emerge in clinical practice. This could provide the opportunity to measure not only troponin levels but also protein modifications of sarcomere proteins that may correlate with disease.^{85,86}

Numerous other clinical assays of proteins are useful in the management or assessment of heart failure, inflammation related to heart disease risk, or cardiac fibrosis. These are well described in excellent review articles.^{87,88} There is interest in further developing biomarkers for vascular disease. For example, transforming growth factor- β isoforms and C-reactive

protein have recently been shown to be potentially useful markers in vascular Ehlers-Danlos syndrome.⁸⁹ Finally, biomarkers of brain injury from stroke are of interest and may be useful in clinical settings to assess ischemic and other forms of brain injury and to stratify treatments.

Despite much progress by directed research and assay development for biomarkers of heart disease, proteomics is likely to be a useful conduit for further biomarker development. It is worthwhile at this juncture to review technologies that will likely drive non-hypothesis-based opportunities for progress in developing new biomarkers or more sensitive and accurate detection of low-abundance peptides and proteins in body compartments, particularly blood. The recent American Heart Association scientific statement on cardiovascular proteomics provides an in-depth review of emerging technologies.⁸⁰

The technology that has driven recent advances in proteomics is MS. A mass spectrometer is an instrument that ionizes, separates by mass-to-charge ratio, generally by acceleration and deflection by electric or magnetic fields, and detects the ionized peptides or chemicals. From this information, the mass of a peptide can be used to infer its identity or in tandem MS (MS/MS or MS2) can reveal its precise amino acid sequence and posttranslational modifications. The output in terms of a spectrum, which displays peaks with relative abundance versus mass-to-charge ratio, can be analyzed against databases to determine the match of a peptide or set of peptides to the protein from which it is derived.

Key steps in applying MS analysis of tissue or body fluids include proper sample collection and storage and separation of complex protein mixtures. Sample preparation involves solubilizing tissue samples. In the case of body fluids, this may involve initial separation steps, for example, to prepare serum or plasma from whole blood. In addition, serum/plasma contains a high abundance of albumin resulting in a very high dynamic range of protein concentrations that limit assessment of low-abundance proteins. Thus, many proteomic approaches used to analyze serum/plasma involve removing albumin. Once the initial preparation is complete, the protein mixture is typically subject to enzyme digestion (eg, with trypsin) to generate peptides. The peptides are separated by physical means such as capillary electrophoresis or liquid chromatography before injection into the mass spectrometer. After analysis of various sample fractions by MS, the resulting data sets are quite complex. Database searches, inspection of primary spectra, and further analysis are necessary before comparisons between samples are undertaken. Refinements in the separation technology and increased sensitivity of MS instruments have allowed highly accurate quantification of proteins in complex mixtures with labeled (mass tags) or unlabeled approaches to compare the quantity of peptides/proteins between groups. The various methodologies for analysis and quantification in proteomics are discussed in detail in the recent American Heart Association scientific statement.⁸⁰

There are numerous barriers to the widespread application of proteomics to the development of disease biomarkers, including the complexity of use of MS-based technologies and each step of the investigative process from sample collection, storage, and preparation to separation of complex mixtures

and MS/MS approaches to final data annotation and analysis. Some of these complexities and pitfalls were illustrated by the outcomes of the Human Proteome Organizations human plasma proteome project, which was a joint effort of multiple laboratories using different approaches.^{90,91} An additional limitation to clinical application of proteomic approaches is that the instruments are very expensive, are challenging to maintain, and require technical staff with a very high level of expertise. Furthermore, the data are challenging to interpret and analyze in group comparisons. In general, the approaches do not currently produce results in a time frame that would allow them to inform clinical care. Finally, although strides have been made to develop methodologies for absolute quantification in comparisons between sample sets, with the use of both labeled and unlabeled approaches, this remains an ongoing challenge.

The translation of a significant and highly accurate biomarker discovered by proteomics to clinical care still requires additional validation in large population studies, and this might involve transition to a validated immunoassay. Two recent articles from conferences of experts point out the requirements for translation of biomarkers discovered with proteomic approaches to useful clinical assays.^{82,92} The recent publication of draft maps and databases of the human proteome may accelerate progress in proteomic application to clinical medicine.^{93,94}

Despite these challenges, application of unbiased proteomics approaches to characterize new biomarkers offers promise in cardiovascular disease. For example, proteomic approaches may help to identify biomarkers to fill current gaps such as the diagnosis of subclinical or chronic brain injury and ischemia, as well as differentiation of distinct forms of stroke. They could enable more accurate prediction of risk for acute MI or stroke before the event. For example, Prentice and colleagues⁹⁵ used an unbiased MS-based approach with enrollment samples from the Women's Health Initiative Observational Study to screen for protein biomarkers for risk of future coronary heart disease and risk of future stroke; they identified β -2 microglobulin and insulin-like growth factor-binding protein 4, respectively. Similarly, Yin and colleagues⁹⁶ used an MS-based approach with samples from the Framingham Heart Study to identify single and combinations of novel protein biomarkers associated with future MI or atherosclerotic cardiovascular disease.

The assessment of risk for SCD to more precisely stratify use of ICDs or even to identify people early enough to avert the event is a substantial unmet need that proteomics may address. Although some tests are available to monitor patients with heart transplantation for rejection, intermittent myocardial biopsy remains the gold standard. The development of new highly sensitive and specific blood-based assays would be a major advance in this field. Finally, there are no available biomarkers to assess risk for aortic dissection pulmonary hypertension severity and response to therapy or that are specific for pulmonary embolism.

Assays developed in an unbiased fashion also have the particular advantage that they may uncover novel mechanisms leading to new or rationally prescribed therapeutics. Proteins and their posttranslational modifications are

the ultimate drivers of function, and from this perspective, advances in proteomics are essential to the development of precision medicine.

Metabolomics

Metabolomics, or metabolomic profiling, refers to a comprehensive analysis of low-molecular-weight (typically <1500 Da) molecules called metabolites. These molecules either are endogenous products resulting from chemical processes in the body (ie, metabolism) or come from exogenous sources (eg, diet, drugs, xenobiotics, or gut-host cometabolism). The metabolome represents the full collection of metabolites in a biological entity such as a cell, tissue, organ, or organism. In humans, metabolites have often been measured in blood or urine, but they can also be detected in saliva, breath, or any of the \approx 500 different histological cell types in the human body.⁹⁷

Metabolomics provides a biochemical fingerprint of an individual at a given point in time. In principle, this should give information about underlying physiological and pathological states that can be linked to disease presence, severity, and prognosis. Recent improvements in analytical technologies and advances in bioinformatics methods enable metabolomic profiling in hundreds or even thousands of individuals in a reasonably short time frame, allowing the broader implementation of this methodology in epidemiological and clinical research. This emerging technology provides unprecedented opportunities to obtain objective measures of biochemical processes reflecting environmental and other exposures in humans. Hence, there has been substantial recent interest in metabolomics in the cardiovascular field.⁹⁸

The 2 main technologies used for metabolomic profiling are nuclear magnetic resonance (NMR) spectroscopy and MS-based methods. NMR spectroscopy uses the magnetic properties of certain atomic nuclei, and it provides unique and well-resolved spectra that are highly predictable for small molecules such as metabolites. As described in the previous section, MS relies on ionization of the sample and subsequent measurement of the mass-to-charge ratio, in which the spectra show relative abundance of the detected ions as a function of mass to charge, allowing identification of unique molecules in the sample. Typically, MS-based metabolomics also involves an initial step in which molecules in the sample are separated by certain chemical properties (eg, charge, size, or polarity) with capillary electrophoresis or, more often, chromatography. In chromatography, the mobile phase can be gas (gas chromatography) or liquid (liquid chromatography).⁹⁷ Advantages of NMR spectroscopy include high reproducibility, minimal sample preparation, no sample destruction, and a relatively low cost per sample. A major disadvantage is a detection limit in the submicromolar range, meaning that fewer metabolites can be detected than with MS-based methods. The MS-based methods, on the other hand, are more sensitive, allowing detection of a larger number of metabolites. However, they are more susceptible to variability, leading to lower reproducibility and higher platform dependency. In addition, the per-sample cost is higher, and the bioinformatics analyses of the generated data are more complex.

Metabolomic profiling can be performed in a targeted or untargeted manner. The targeted approach aims at accurately

measuring a specific subset of metabolites, typically focusing on a certain pathway or group of molecules of interest. The advantages of this approach include that the use of internal standards allows quantitative interpretation and that the optimized sample preparation leads to fewer analytical artifacts, improving downstream analysis. The untargeted approach aims at a comprehensive analysis of all measurable analytes in the sample and unknown compounds. This approach allows novel target discovery and avoids restricting the study to a certain group of metabolites, but it is associated with more challenges in the chemical analyses, in downstream bioinformatics, and especially in compound identification.⁹⁹

Several early studies applying metabolomics in cardiovascular disease research used NMR spectroscopy in small samples of patients being investigated with coronary angiography for suspected coronary heart disease. The first of these studies reported very promising results; it achieved sensitivities and specificities >90% for correctly classifying patients with chest pain as having or not having significant coronary stenosis.¹⁰⁰ However, follow-up studies demonstrated weak discrimination for detecting angiography-defined CAD in a patient sample similar to that of the first study¹⁰¹ or in high-risk diabetic patients.¹⁰² In one of the first efforts applying MS-based metabolomics to study cardiovascular diseases, 6 metabolites from the citric acid cycle pathway were found to change significantly more among patients who demonstrated inducible ischemia during an exercise test than among those who did not have signs of ischemia.¹⁰³

One of the larger efforts to use metabolomic profiling to predict cardiovascular diseases was a case-control study of subjects from the Catheterization Genetics biorepository in which 69 metabolites were assessed in 314 cases with CAD and 314 age- and sex-matched controls free of disease.¹⁰⁴ Using principal components analysis, the investigators showed that branched-chain amino acids (primarily leucine and isoleucine) and urea cycle metabolites were independently associated with the presence of CAD. In a separate analysis, they related metabolites to adverse events (death or MI; $n=63$) during 2 years of follow-up and found dicarboxylacetylcarnitines to be associated with subsequent events. In a larger follow-up study of 2023 patients in the MURDOCK (Measurement to Understand Reclassification of Disease of Cabarrus/Kannapolis) Horizon 1 Cardiovascular Disease Study, the same 69 metabolites were analyzed as predictors for death ($n=232$) and a composite end point (death or MI; $n=294$) during a median follow-up of 3.1 years.¹⁰⁵ The strongest metabolite predictors of death or MI among those individuals who had undergone a cardiac catheterization ($\approx 61\%$ had at least 1-vessel disease) were short-chain and long-chain dicarboxylacetylcarnitines and fatty acids. The investigators also demonstrated that the metabolomic profiles added incremental discriminative capacity beyond standard clinical variables in individuals with intermediate risk.

In 2011, investigators from the Framingham Heart Study and the Malmö Diet and Cancer study used untargeted liquid chromatography–MS to study metabolites associated with diabetes mellitus risk. They reported that 5 branched-chain and aromatic amino acids (isoleucine, leucine, valine, tyrosine, and phenylalanine) were highly significantly associated

with future type 2 diabetes mellitus.¹⁰⁶ The 3 amino acids most strongly associated with diabetes mellitus (isoleucine, tyrosine, and phenylalanine) were subsequently shown to predict incident atherosclerotic cardiovascular disease (MI or stroke), carotid intima-media thickness and plaques, and exercise-induced myocardial ischemia.¹⁰⁷ These findings highlight a potential key role of amino acid metabolism in early diabetes mellitus development, and they point to a potential link between type 2 diabetes mellitus and susceptibility to atherosclerotic cardiovascular disease.

Recently, 4 studies applying metabolomic profiling in individuals from the general population to study risk for future atherosclerotic cardiovascular disease were published. Vaarhorst and colleagues¹⁰⁸ used NMR spectroscopy to study associations of 36 metabolites with incident coronary heart disease ($n=79$). They reported that a score of 13 metabolites representing different biological pathways (selected with the use of least absolute shrinkage and selection operator regression) was associated with disease incidence but did not improve risk prediction beyond traditional cardiovascular risk factors. Stegemann and colleagues¹⁰⁹ profiled 685 plasma samples (number of incident events=90) by shotgun lipidomics using MS-based methods to identify 135 lipid species. Triacylglycerols and cholesterol esters with a low carbon number and double-bond content were the strongest predictors of atherosclerotic cardiovascular disease (a composite end point including incident fatal and nonfatal MI, ischemic stroke, and SCD). Inclusion of 3 of these lipid species in a model with traditional risk factors resulted in improved risk discrimination and reclassification. Ganna and colleagues¹¹⁰ applied untargeted MS-based metabolomics in 2698 individuals (with 413 incident events of coronary heart disease) and reported 4 metabolites that could be replicated and were independent of major cardiovascular risk factors (lysophosphatidylcholine 18:1, lysophosphatidylcholine 18:2, monoglyceride 18:2, and sphingomyelin 28:1). Together, these 4 metabolites contributed to moderate improvements in discrimination and reclassification in addition to traditional risk factors. Monoglyceride 18:2 showed an enrichment of significant associations with genetic variants known to be associated with coronary heart disease, as well as the suggestion of a causal relation with coronary heart disease based on mendelian randomization analysis. Würtz and colleagues¹¹¹ used NMR spectroscopy to assess 68 lipids and metabolites in targeted profiling of 13441 individuals (including 1741 incident cardiovascular events) from 3 large cohort studies. When adjusted for traditional cardiovascular risk factors, including routine lipids, higher serum phenylalanine and monounsaturated fatty acid levels were associated with increased cardiovascular risk. Higher omega-6 fatty acids and docosahexaenoic acid levels were associated with lower risk. Risk classification with a score of these 4 metabolites was particularly improved for people in the intermediate risk range, although discrimination was not enhanced.

Although stroke was included as part of a composite cardiovascular end point in some of the above metabolomics studies, there are relatively few studies applying metabolomic profiling to study stroke specifically. After a few smaller case-control studies of cerebral infarction indicated good

separation of cases and controls with multivariate statistical analyses.^{112,113} Jové and colleagues¹¹⁴ performed untargeted metabolomics using liquid chromatography–MS in 293 patients with transient ischemic attack to find metabolites associated with worse outcome. Low concentrations of lysophosphatidylcholine 16:0 were significantly associated with stroke recurrence, whereas 2 other lysophosphatidylcholines (20:4 and 22:6) also showed potential as biomarkers for other signs of worse outcome.

Although most studies using metabolomic profiling to study heart failure have focused on finding diagnostic markers in symptomatic patients, a few studies have used metabolomics to find novel predictive or prognostic markers for heart failure.¹¹⁵ Zheng and colleagues¹¹⁶ reported dihydroxydocosatrienoic acid and hydroxyleucine to be associated with incident heart failure in 1775 individuals (number of events=276) from the general population assessed with MS-based metabolomics. Desmoulin and colleagues¹¹⁷ used NMR spectroscopy to find prognostic biomarkers in 200 patients with acute heart failure, and they reported that lactate, cholesterol, and the lactate-to-cholesterol ratio predicted 30-day mortality. Cheng and colleagues¹¹⁸ performed MS-based metabolomic profiling in 401 patients with heart failure and showed that a panel of metabolites (asymmetric methylarginine/arginine ratio, butyrylcarnitine, spermidine, and total amount of essential amino acids) provided prognostic value independently of B-type natriuretic peptide and other established prognostic factors.

As may be evident from the above, although some classes of metabolites seem to come up as being associated with atherosclerotic cardiovascular disease in many studies such as branched-chain amino acids, lysophosphatidylcholines, and fatty acids, there is substantial heterogeneity, even across studies with similar designs, techniques, and outcomes. The reasons for the discrepancies are likely several. As in any biomedical study, slight differences in methods can translate to substantial dissimilarities in results and interpretation. This is especially true for new high-throughput methods for which there is ongoing, often intense, methods development in parallel with applied studies and in which small changes in the settings of the instrument and bioinformatics methods may give an entirely different set of results and hence are highly relevant for metabolomics, in particular MS-based metabolomics. Apart from continued efforts to standardize analytical techniques and bioinformatics approaches, other ways to move the field forward are likely to include increased collaborative efforts. This would include meta-analysis of results from different analytical platforms to increase sample sizes and generalizability of results and increased open data sharing. Other possible future directions for cardiovascular investigators using metabolomics are increased efforts at data integration, for example, combining metabolomics with genomics¹¹⁹ or transcriptomics¹²⁰ to use different data layers for improved biological understanding of pathways leading to cardiovascular diseases.

Peripheral Cell Analysis

To further take advantage of the expressed genome in the diagnosis, prognosis, and therapeutic decision making for patients with cardiovascular diseases, accessing cell population

subsets and interrogating them for the expressed genome are gaining acceptance and have a few notable advantages. First, one can focus on selected cell populations that may be more relevant for the disease of interest than analyzing an admixture of a variety of cell types (the focused profile). Second, the circulating cell population, particularly immune cell subsets, may contain enriched mirrored information pertaining to areas of injury or inflammation, thus providing insight into diseased tissues (the surrogate biopsy). Finally, specific cell populations of interest may be extracted and propagated under precise environments to provide a more controlled systems biology profile, creating a platform of a “disease in a dish.”

In addition to the analysis of expressed genes in cells, there is recent enthusiasm for the systematic analysis of microRNAs in the blood, which are remarkably stable in circulation and may function as disease biomarkers.⁵⁶ MicroRNAs normally function to regulate gene expression and can potentially be delivered to target cells to ectopically regulate genes. Similarly, one could also use proteomic approaches to identify changes in expressed protein abundance, or protein modifications, as part of the profiling to characterize the pathophysiological process. It has been suggested that a combined approach, for example, integrating transcriptomic and proteomic data, will ultimately be the most useful for the understanding of disease pathways.¹²¹ Through combinatorial analyses, one may be able to understand the precise interactions of susceptibility, environmental epigenetic regulation, and functional protein production to influence clinical outcomes.

In terms of cell source, the most direct means to study cardiovascular diseases is cardiac and vascular biopsies. The retrieved cells can be phenotyped by immunostaining or extracted, for example, by laser capture, and analyzed for profiles related to the expressed genome. The cells can thus be sources for mRNA, microRNA, proteomic, or metabolomic profiles. For example, the evaluation of gene or protein profiles is used for the diagnosis of cardiac amyloidosis.¹²² However, obtaining any of these types of samples requires invasive techniques. In situations in which the disease process is systemic, analysis of more readily accessible peripheral tissues such as skin or fat cells can be very informative.

The bloodstream provides a noninvasive source of cells to examine the expressed genome. The expression profiles of circulating immune cells, including innate immune (macrophage and dendritic cells) and acquired immune (T and B cells) populations, often reflect the internal biological environment of the host, particularly under stress conditions. An early study of 41 patients with CAD demonstrated that the gene expression analysis of peripheral blood mononuclear cells identified 14 unique genes with an expression that reflected the actual severity of coronary disease.²⁷ This was replicated in a second cohort of 107 patients, consistent with the notion of a potentially important interplay of inflammatory cell gene expression and coronary atherosclerosis.

Supporting this concept further was a recent study involving 24320 individuals seeking novel risk markers for atherosclerotic coronary disease. The study identified a number of recessive genes that show “runs of homozygosity” in association with CAD. These genes are a coordinated subset of 44 mRNAs expressed in monocytes and 17 mRNAs expressed

in macrophages, suggesting again that changes in the gene expression in these key inflammatory cell types can contribute to the pathogenesis of atherosclerosis.¹²³

Further analysis and phenotyping of innate immune cells such as macrophage and dendritic cells, whether in the circulation or from local tissues, can reflect the changes in the expressed genome and evolving polarity in different settings of tissue injury. Indeed, within the immune cell population, the gene expression and cytokine production patterns can differentiate between the proinflammatory and regenerative subsets, often referred to as the M1 and M2 macrophage polarization states, respectively. In human atherosclerotic plaques, M1 proinflammatory macrophages were found to dominate in the rupture-prone shoulder regions of the plaques, and the more protective M2 population was located in stable portions of the plaques and the adventitia of blood vessels.¹²⁴

Similar to the example of the role of the expressed genome in atherosclerosis, efforts have been extended to understanding obesity and cardiometabolic risk in patients. Gene and protein expression analyses of fat and inflammatory cells in obese diabetic patients have been performed. Mraz and colleagues¹²⁵ examined inflammatory cytokine profiles and mRNA expression in adipose tissue and macrophages in 20 obese women compared with 15 control subjects and found increased expression of chemotactic and proinflammatory factors such as CD68, CCR-1, CCR-3, CCR-5, and Toll-like receptors 2 and 4 among the obese patients. A 2-week intervention with a very-low-calorie diet led to a significant decrease of chemokine receptors in the harvested macrophages and decreased chemokines in the adipose tissues. This might point to an inflammatory signaling network associated with the metabolic syndrome and the plasticity of the expressed genome in response to environmental changes. A different intervention, bariatric surgery, is known to decrease the presence of diabetes mellitus and its complications over time in morbidly obese patients, although the mechanism is unclear. Poitou and colleagues¹²⁶ analyzed RNA expression profiles directly from adipose tissue in 22 obese women before and 3 months after bariatric surgery. Their data suggested a major downregulation of innate and adaptive immune pathways and a reduction in interferon signaling to be potentially responsible for the improvement in clinical outcomes.

A parallel approach has been to perform peripheral mononuclear cell proteomics to define the expressed protein differences between obese and lean patients. The investigators found that among obese patients, there were increases in proinflammatory signals such as thrombospondin-1, a glycoprotein with proinflammatory and antiangiogenic properties.¹²⁷ On the other hand, histone deacetylase 4, an epigenetic anti-inflammatory regulatory protein, was downregulated among obese patients. Intriguingly, these abnormal protein profiles, although suggesting an overactive inflammatory response, could be significantly reversed by exercise.

Because the bulk of the expressed genome in whole blood represents contributions from circulating cells, one may gain glimpses into biological processes or disease pathways by examining the whole blood for expressed genes or proteins rather than specific cell types. For example, Peters and

colleagues¹²⁸ recently examined the transcriptomic patterns associated with aging by performing a whole-blood gene expression meta-analysis of 14983 individuals. The differentially expressed genes reflected changes in metabolic function, transcriptional and translational dysfunction, immune senescence, and mitochondrial decline. These transcriptomic changes were in turn associated with cardiovascular risk phenotypes such as increases in blood pressure, cholesterol levels, fasting glucose, and body mass index.

An interesting consequence of inflammatory cell load and activation, reflecting disease activity, is that with increased inflammation, there is associated shortening of leukocyte telomeres.¹²⁹ O'Donovan and colleagues¹³⁰ found in a cohort of 1962 adults in their eighth decade that leukocyte telomere length was strongly associated with level of interleukin-6 and tumor necrosis factor- α release. This likely reflects the accelerated aging process and higher turnover of the immune cells in the setting of increased inflammation. Studies by Epel and colleagues¹³¹ in the MacArthur Health Aging Study found that the rate of telomere length change in leukocytes predicts overall mortality and is a strong independent prognostic factor for survival.

Finally, peripheral blood cells or cells from other tissue sources may be reprogrammed to become induced pluripotent stem cells suitable to model certain aspects of disease by incorporating the individual genetic susceptibility together with molecular and functional changes associated with disease phenotype.^{132,133} These cells may be differentiated into myocardial or vascular lineages to permit detailed studies of the expressed genome. This technology can provide both insights into the disease phenotype and a platform from which to derive innovative biomarkers and to test therapeutic interventions in a patient-specific manner.¹³⁴ The induced pluripotent stem cells may also be differentiated into mononuclear cell-derived macrophages to model, for example, cellular polarization in disease states. This has been applied in the analysis of genetic conditions such as Tangier disease to provide biological insights through changes in the expressed genome in differentiated Tangier disease macrophages compared with control macrophages.¹³⁵

Although expressed genome profiling of peripheral cells has advanced our biological understanding of cardiovascular diseases and enabled more sophisticated research studies, its translation into clinical practice has been much slower. As described in an earlier section, one area of clinical utility has been the detection of cardiac transplant rejection after surgery. Based on changes in whole blood gene expression profiles, a rejection score has been developed using a number of genes presumably involved in T-cell and natural killer cell activation and stem cell mobilization in the bloodstream. The rejection score was evaluated in the IMAGE trial (Invasive Monitoring Attenuation Through Gene Expression), which showed that gene expression profiling was noninferior to routine biopsies between 6 and 60 months after transplantation.⁵⁵ A more recent trial evaluated the same scoring tool among patients at a much earlier posttransplantation stage, between 55 days and 6 months. The results also showed that gene expression profiling was noninferior to biopsies while providing guidance with respect to corticosteroid weaning for the patients being thus monitored.¹³⁶

Molecular Imaging

Analysis of the expressed genome in whole blood or specific circulatory cells provides a general systemic readout of changes in RNA or protein levels in response to external stress predicated by the genetic background of an individual. However, the information does not necessarily provide information on the geographic localization or the extent of involvement of the injured or stressed organs or tissues. To take precision medicine to the next level of personalization and localization, it would be ideal to be able to characterize the degree of molecular expression in specific organs or tissues of interest and to quantify the changes over time or in response to treatment.

The ability to tag specific molecular entities such as proteins, drugs, or metabolic substrates with imaging-detectable tracers has raised the possibility of evaluating the expressed genome directly *in vivo* in patients. The tracers available for imaging can include positron-emitting labels (eg, C-11, N-13, F-18, Cu-64, Zr-89) suitable for positron emission tomography (PET), gamma-emitting labels (e.g., Tc-99m, In-111, I-123), or paramagnetic (gadolinium, C-13)/ferromagnetic (iron oxide) labels for magnetic resonance imaging. The ability to label specific protein species, drugs, and metabolic substrates permits the tracking of these injected, inhaled, or ingested moieties in patients in terms of localization, quantification, and kinetics of specific products of the expressed genome.

One example of abnormally expressed proteins that can be detected by molecular imaging occurs in cardiac amyloidosis. Increasingly, mutations of the transthyretin protein, which is made in the liver to transport thyroxine and retinol, have been recognized to lead to protein misfolding and aggregation. Similarly, in patients with multiple myeloma, abnormal light chains can also accumulate in the heart. The deposition of abnormal amyloid fibrils in the myocardium can lead to restrictive infiltrative cardiomyopathy with a median survival of ≈ 2 years.¹²² Traditionally, the abnormal mutated proteins have been identified on myocardial biopsy with special staining or protein electrophoresis or sequencing. Recently, [F-18]-florbetapir, a specific amyloid protein-labeling, positron-emitting tracer approved for amyloid imaging in the brain, has demonstrated specificity in identifying cardiac amyloid deposition in patients with amyloidosis in a proof-of-concept pilot study.¹³⁷ In the future, the amyloidogenic protein may be detected first through noninvasive imaging strategies, complementing and possibly replacing the myocardial biopsies currently in use to make the diagnosis.

[F-18]deoxyglucose is a positron-emitting glucose analog that can delineate cellular uptake and metabolism of glucose in the tissues and organs. In addition to the myocardium, inflammatory cells such as activated macrophages can take up significant amounts of label. With the appropriate suppression of glucose uptake in the myocardium, one can identify localized inflammatory processes in the atherosclerotic plaque or myocardium. In particular, [F-18]deoxyglucose imaging may provide an early diagnosis of atherosclerosis, especially with respect to active vulnerable plaques that are prone to rupture in the carotid or coronary circulation.¹³⁸ Furthermore, [F-18]deoxyglucose PET has proven to be valuable in identifying

inflammatory processes in the myocardium, for example, sarcoidosis. This has been very helpful in identifying inflammatory cell localization and severity in patients suspected of having sarcoid heart disease and in allowing the monitoring of response to therapy.¹³⁹

To more specifically detect vulnerable plaques, Müller and colleagues¹⁴⁰ compared the levels of matrix metalloproteinases (MMPs), proteolytic enzymes expressed by local inflammatory cells, in vulnerable plaques removed by carotid endarterectomy and control samples. MMP-1, MMP-9, MMP-12, MMP-14, and CD68 were all increased in vulnerable plaques. An [F-18]-labeled MMP-2/MMP-9 inhibitor specifically targeting activated MMPs was successful in labeling atherosclerotic plaques but did not distinguish vulnerable plaques from stable plaques with high specificity. An alternative strategy is to use [F-18]-labeled folate to detect folate receptor β , which is overexpressed in activated macrophages in the inflammatory plaque and has been found to concentrate in vulnerable plaques removed by carotid endarterectomy.¹⁴¹ A hybrid approach combines PET with computed tomography to identify the dynamics of calcium deposition in active plaques. This can be accomplished by [F-18]-labeled sodium fluoride imaging with PET to identify patients with coronary plaque vulnerability associated with increased cardiac events, together with calcium score assessed by computed tomography.¹⁴²

Protein ligands or small-molecule moieties or drugs can be labeled and used to detect the specific localization and quantitative expression of specific cell surface receptors and potential changes under stress or injury. Using this technology, one could detect type I angiotensin receptor using [C-11]methyllosartan or [In-111]angiotensin for PET or radionuclide imaging quantification, respectively.¹⁴³ Conversely, one could label inflammatory cells with lysosomal degradation substrates with iron particles so that the process is detectable through magnetic resonance imaging. A more targeted approach has been the use of magnetization-susceptible labeling of vascular cell adhesion molecule-1, which has been effective in detecting the inflammatory changes in both core and penumbral regions in the brain after middle cerebral artery occlusion in a mouse model of stroke.¹⁴⁴

Molecular imaging of the expressed genome is still very early in its development. In contrast to high-throughput techniques now available for transcriptomics, epigenomics, proteomics, and metabolomics, molecular imaging labeling to date delineates only 1 or very few selected targets at a time. In the future, the use of multiple labels of different energies and the increased availability of hybrid imaging modalities such as PET-computed tomography and PET-magnetic resonance will expand the repertoire of tools for molecular imaging of the expressed genome.

Conclusions

Transformational technologies paired with the completion of the Human Genome Project have unleashed an unprecedented wave of omics studies that promise not only to deepen our understanding of cardiovascular diseases and stroke but also to facilitate a much improved ability to diagnose, predict, and prognosticate diseases in individual patients, a key goal of precision medicine. We now have the ability to address

disease at many levels that were inaccessible to us during the past century: the genome, transcriptome, epigenome, proteome, metabolome, cells, tissues, and organs. Each of the omics approaches remains a work in progress, as outlined in this statement, and many of the initial findings are still awaiting systematic replication in independent studies. Nonetheless, we can expect the next 5 to 10 years to witness enormous progress in the application of the approaches to the study of specific cardiovascular diseases in patients. A critical step to facilitate the eventual widespread use of the expressed

genome in the clinic is funding of large-scale efforts to validate, replicate, and integrate the information streams arising from various omics studies such as the National Heart, Lung, and Blood Institute's Trans-Omics for Precision Medicine Program. As such efforts come to fruition, we can expect a host of novel disease biomarkers to emerge and find application in the clinic, as well as a new push to understand the interactions of genetics and environment in disease pathogenesis. For cardiovascular investigators, one can scarcely imagine a more exciting time to be engaged in translational research.

Disclosures

Writing Group Disclosures

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*Modest.

†Significant.

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*Significant.

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The Expressed Genome in Cardiovascular Diseases and Stroke: Refinement, Diagnosis, and Prediction: A Scientific Statement From the American Heart Association

Kiran Musunuru, Erik Ingelsson, Myriam Fornage, Peter Liu, Anne M. Murphy, L. Kristin Newby, Christopher Newton-Cheh, Marco V. Perez, Deepak Voora and Daniel Woo on behalf of the American Heart Association Committee on Molecular Determinants of Cardiovascular Health of the Council on Functional Genomics and Translational Biology and Council on Epidemiology and Prevention; Council on Cardiovascular Disease in the Young; Council on Cardiovascular and Stroke Nursing; Council on Cardiovascular Surgery and Anesthesia; Council on Clinical Cardiology; and Stroke Council

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