Advances in Genetics, Proteomics, and Metabolomics

Progressing From Risk Factors to Omics

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Many factors contribute to the development of clinical atherosclerotic disease in adults. Observational studies conducted from the late 1940s onward have helped us to identify cigarette smoking, cholesterol levels, diabetes, blood pressure, and other factors as important determinants of cardiovascular disease (CVD) outcomes.1,2 The effects are multiplicative. For example, smoking, hypertension, and diabetes each double the risk of a heart attack, and having all 3 factors can lead to a risk that was ≈8 times that of an adult of similar age and sex who did not have any of the factors.3

A major focus in the earlier population and clinical studies was identification of persons at increased risk before the clinical atherosclerotic event. Less attention has been directed toward other types of events, such as occurrence of a myocardial infarction in persons presenting with chest pain, recurrent CVD events, and long-term vascular disease complications such as cardiac failure. The determinants of these vascular events are likely to include some of the classical risk factors, but new factors will need to be important as well.

Improvements in outpatient and inpatient care, diagnosis, and biomarker discovery have reshaped the landscape. It is possible to visualize subclinical CVD with modern atherosclerotic imaging techniques such as carotid intima-media thickness and arterial calcification assessments. Clinical chemists and geneticists identify new biomarkers that promise to help refine assessments of CVD risk, but it is not clear how such tests should be used for research or for clinical care. This article addresses several of the questions posed above and takes the perspective of research and care for clinical atherosclerotic disease, emphasizing the progress we have made from traditional risk factor assessment to the present time that includes newer assessment methods such as genomics, metabolomics, and predictive medicine.

Traditional Risk Factors for Cardiovascular Disease

The traditional risk factors include cholesterol, blood pressure, diabetes mellitus, smoking, age, and male sex. Understanding progress in this field serves as a good starting point for evaluation of newer biomarkers of disease. First, observational cohort studies that largely included persons not on medications who were followed for a decade or more were commonly used to assess the risk of developing a first vascular disease event such as a myocardial infarction, angina pectoris, or coronary death. In the current era, the use of cholesterol and blood pressure lowering medications has become more common and the assessment of factors that might alter risk for a vascular event has become more complicated. Second, the severity of the first vascular disease event has generally decreased over the last 40 years, as coronary mortality has declined and the importance of specific risk factors such as smoking may have changed. Third, screening an individual to assess risk is best understood from the perspective of groups, not individuals. For example, risk of a first CVD event can be estimated from several items: history (age, sex, smoking, and medications used), physical examination (blood pressure), and laboratory values (cholesterol, high-density lipoprotein cholesterol, and fasting glucose for diabetes assessment). An element or error is associated with all risk estimates, and this fact is often not appreciated by users of these estimates.

The relative effects of traditional risk factors differ according to the vascular disease outcome being studied. For example, peripheral artery disease is highly associated with smoking and diabetes mellitus, and the other traditional risk factors are less important in causing arterial blockages and intermittent claudication.4,5 Similarly, congestive heart failure is highly associated with hypertension, history of myocardial infarction, and history of valvular heart disease.6 Traditional risk factors have also been shown to be determinants of coronary artery vascular calcification for asymptomatic adults in large cohort studies.7,8

Progression of atherosclerosis over time is relatively understudied. Figure 1 presents a schematic of the different stages of atherosclerotic disease. Subclinical assessments can include vascular calcification (several sites possible) and vascular physiology (pulse wave velocity, flow-mediated vasodilation, and others). There are many opportunities to investigate the determinants of disease progression, with clear descriptions of the findings at baseline and findings at follow-up (subclinical or clinical outcomes) outcomes. Such an approach was used in the Multi-Ethnic Study of Atherosclerosis, where the investigators demonstrated the importance of coronary artery calcification as a determinant of clinical coronary heart disease events.9

The medical statistician Bradford-Hill summarized criteria used to assess associations between a disease and a supposed causative agent (Table 1).10 Traditional risk factors fulfill...
these criteria and, from the perspective of atherosclerosis research, it is important to emphasize a few key issues. First, temporality is important and the risk factor being assessed should be measured before the development of disease. For that reason, prospective studies are needed to make definitive statements about etiology, and case-control or cross-sectional studies are generally not adequate. Second, demonstration of a biological gradient, plausibility, coherence, and experimental data requires multiple investigations undertaken in different environments.

**Novel Risk Factors for Cardiovascular Disease**

New risk factors for CVD are constantly being evaluated. It is important to develop an approach to assess the research and clinical utility of such measurements. Some factors, especially if considered individually, meet the A. B. Hill criteria as shown in Table 1, but they might improve on our current approach. Discussion of the merits of a new risk factor or biomarker for CVD necessarily includes clinical utility. Manolio has suggested that new measures should add independent information about the risk or prognosis, it should account for a large proportion of the risk associated with a given disease or condition, the measure should be reproducible, and that measures used as diagnostic tests should be sensitive, specific and have a high predictive value. Mosca emphasized slightly different issues and asked health care providers to consider 10 questions before incorporating newer factors into clinical practice. These tenets included standardization, prognostic value, clinical management, test specificity, test sensitivity, effects of interventions, traditional risk factor burden, medical treatment, overall benefit, and cost.

Table 2 updates the novel risk factor criteria discussed by Manolio and Mosca. The key criterion is listed first—the new factor adds (or effectively replaces) information concerning risk or prognosis for a specific clinical or subclinical outcome. The addition of useful information does not necessarily mean that a new test would be used in the entire population. Although screening programs and risk estimation have generally used statistical methods that discriminate between future cases and noncases for a specific outcome, newer risk assessment methods provide for the use of a multivariable estimate of risk with traditional risk factors and critical evaluation of the potential inclusion of a new risk factor into the formulation, as developed by Pencina et al. The new method of reclassifying individuals helps to identify the subset of individuals for whom the new test might be most useful. Such methods build on previous experience with discrimination methods that used subgroups in initial analyses and the use of stepwise regression methods. The traditional methods and the newer methods are complementary. In fact, experts in the field are now recommending that several statistical measures should be reported when evaluating the utility of a new biomarker. An important concept in the first criterion is that a biomarker can help assess “risk or prognosis.” Biomarkers do not necessarily need to contribute to both risk estimation (assessment in a screening environment) and prognosis (assessment of a factor for a person on therapy). As an example, total cholesterol levels are very helpful as part of an evaluation of risk for developing an initial coronary heart disease event and low-density lipoprotein cholesterol (LDL-C) information, in the context of screening, does not provide much of an advantage over a total cholesterol determination. However, LDL-C or other lipid biomarkers such as non–high-density lipoprotein cholesterol, apolipoproteins, or the newer lipid subclasses may be useful to guide therapy and to determine prognosis, and such evaluations are very active area for scientific inquiry at this time.

Equivalence is an important concept in the assessment of new biomarkers. Much experience has been gained with clinical trials concerning equivalence, but there has been little research concerning biomarker equivalence. For example, should we measure LDL-C levels as a predictor of coronary disease or should we use apolipoprotein B? Well constructed prospective studies have generally shown that apolipoprotein
B has excellent predictive capability for coronary heart disease and can be measured reliably, but clinicians have little experience with the measurement and a well-established apolipoprotein B laboratory standardization program is not in place at the present time. The real question is not whether we would measure both of these biomarkers. Rather, the issue is whether clinicians and researchers are ready to move to the measurement of apolipoprotein B instead of LDL-C, and such determinations may be especially relevant for persons with diabetes mellitus.

Another criterion for assessment of a new biomarker in the population setting is that a significant proportion of the risk ought to be attributable to marker (Table 2). This concept favors the use of biomarkers that are relatively common in the population and have an effect on risk. A different perspective is obtained when selected groups are being investigated or surveyed. For example, a new biomarker may be very helpful in determining risk or prognosis in a family or a group that is affected by premature vascular disease, but the biomarker may be relatively uncommon or not as useful in the population at large. Reproducibility and standardization of assays is important to gain widespread use of a test, the performance characteristics (sensitivity, specificity, predictive value) of the test need to be considered, and the clinical utility of an abnormal test result should be considered as well. Finally, the cost of a test is of some importance, but it should be realized that the costs of new tests can often be reduced greatly with automation and adaptation of the test to different ways to make the measurements in the laboratory.

Progress in Genetics, Lipids, and Atherosclerotic Disease

Abnormal blood levels of cholesterol are important determinants of increased risk for CVD, and there are several well-recognized genetic variants that are associated with premature atherosclerosis. Some of these lipid abnormalities are associated with clinically recognizable phenotypes with skin lesions such as xanthomas and xanthelasma, and abnormalities include familial hypercholesterolemia, defective apolipoprotein B, sitosterolemia, and recessive forms of hypercholesterolemia. The discovery of genetics variants that are related to levels of lipoproteins has increased greatly during the past few years. Many genetic variants have been associated with increased concentrations of LDL-C, an analyt e that was first assayed using ultracentrifugation methods developed at the Donner Laboratories and at the National Institutes of Health. Lipoproteins were classified according to the buoyancy of the particles, and later methods showed that precipitation of apolipoproteins, electrophoresis, and magnetic resonance properties, and oxidizability of the particles could also be used to further characterize these lipoprotein markers in plasma. This detailed characterization of the different protein moieties using a variety of techniques would fit the modern definition of lipid proteomics.

Reliable lipid measurement to assess the role of oxidized plasma lipids as a risk factor for atherosclerosis, and developing reproducible assays for oxidized LDL particles has been more problematic. Investigators have shown that higher concentrations of oxidized LDL particles in fasting individuals are associated with greater risk of clinical CVD. Other research shows that antibodies are developed against LDL particles, and that it is important to measure both oxidized LDL and the LDL-antibody complexes.

Both fasting and nonfasting lipid measures are predictive of vascular disease events, and consumption of a high-fat meal has been shown to be highly associated with abnormal brachial artery vasodilation in the postprandial state. These physiological changes occur with little change in total cholesterol, and there is mounting evidence that a variety of oxidized lipids, free fatty acids, markers of oxidation, and inflammatory biomarkers increase after eating. More complete characterization of the lipoprotein, metabolic, and biomarker changes after eating poses new challenges for atherosclerosis researchers. It is not known at this time whether these newer measures will improve the ability to assess risk for developing CVD when screening populations or if such information will help to determine the prognosis for persons being treated to prevent CVD events.

Apolipoprotein E (ApoE) exemplifies a protein that has been linked to premature atherosclerosis and lessons have been learned concerning the genetics, lipid levels and their response to diet and medications, and clinical outcomes. ApoE defects and ApoE deficiency in humans has been linked to premature atherosclerosis and specially bred ApoE knockout mice have been used as an animal model for premature atherosclerosis. The gene that produces ApoE has 3 major alleles: $\epsilon_2$, $\epsilon_3$ and $\epsilon_4$, and there are 6 genotypes: $E2/2$, $E2/3$, $E2/4$, $E3/3$, $E3/4$, and $E4/4$. The $\epsilon 3$ allele is found in ~78% of adults. The $\epsilon 4$ allele, found in 14% of adults, is associated with increased levels of LDL-C, a moderate tendency toward elevated triglyceride levels, and premature coronary artery disease, especially in persons who are ApoE 4 homozygotes. Approximately 9% of the population has the $\epsilon 2$ allele, which is associated with low LDL-C levels, a moderate tendency toward increased triglyceride levels, and mildly decreased risk for atherosclerotic disease. Discovery of new genetic loci associated with traditional lipid levels is accelerating, as evidenced by the recent reports of several new candidate regions that are associated with differences in lipid levels. Some of the findings have already been replicated and follow-up replications and fine sequencing will follow.

The cholesterol and pharmacogenetics study investigated the LDL-C lowering effects of simvastatin, an inhibitor of 3-hydroxyl-3-methylglutaryl-3-coenzyme A reductase, according to the single nucleotide polymorphisms and common haplotypes of the 3-hydroxyl-3-methylglutaryl-3-coenzyme A reductase gene. The authors identified 11 single nucleotide polymorphisms and 10 common haplotypes and reported baseline and LDL-C levels after a 6-week course of simvastatin. Overall, the LDL-C reduction was 3.9% less in blacks, black carriers of the $H7$ or $H2$ haplotype had significantly lower LDL-C at baseline, and black carriers of the $H7$ or $H2$ haplotype had less LDL-C lowering on therapy compared with blacks who did not carry either haplotype. No haplotype effects were reported for whites in this study and the authors concluded that the haplotype effects in blacks were small and not of great clinical relevance, but the results provided further
Integrative Strategies for Biomarker and Genetics Assessment in Populations

A comprehensive review of biomarkers for CVD that was published in 2006 provided directions and signposts for the roadmap of discovery and clinical care related to this field. Since that time further publications have called for greater biomarker discovery to potentially synergize the “complementary power of genetics, transcriptional profiling, and metabolomics.” Identification of new genetic markers that may be associated with altered risk for adult CVD have generally not been as rewarding as might be expected. For example, in the a genome-wide association study of >14,000 cases of 7 common diseases and 3000 shared controls, the Wellcome Trust collaborators reported only one new independent association with coronary artery disease was reported compared with greater success for rheumatoid arthritis, Crohn disease, and type 2 diabetes mellitus. Investigations that use genome-wide association study require large numbers of participants and a great deal of genotyping. Methods to set up these studies have become more formalized to help reduce false-positive results, limitations concerning identification of some types of genetic variants, and selection bias in terms of study participants. We continue to face the difficult issue of validating results for genetic findings when efforts are made to replicate results.

In addition to attempting validation of studies that have found associations between genetic markers and CVD, the appropriate next steps include fine mapping the affected genetic regions with deep sequencing, undertaking new investigations that test for similar results in other population groups, especially in those recognized to be at high risk for the outcomes. Full assessment of the predictive potential will necessarily need to consider the population prevalence of the gene variants, and hopefully, specific interventions might target the genes and the metabolic products of these genes. Such strategies have already been mapped out for type 2 diabetes in adults. In the future, we will especially face the daunting challenge of investigating the more common genes that exert smaller effects and that are associated with >1 trait or disease.

Clinicians hope that genetic and metabolic pathway research will provide improved ways to identify persons with higher risk for adverse outcomes and that “not all risk factors are predictors of ischemic heart disease (or another adverse outcome) in all subsets of individuals.” What is the utility in genetic testing for lipid abnormalities at this time? Humphries et al addressed that question for familial hypercholesterolemia in a recent publication. They noted that >1000 genetic variants now account for this condition. Tendon xanthomas and very high lipid levels may not be present in persons who have molecular evidence of a variant that predisposes the person toward higher cholesterol levels and premature CVD during their lifetime. Selective use of molecular testing within families (cascade testing) can very effectively identify persons with familial hypercholesterolemia using this approach. The affected persons can then be counseled for lifestyle changes and lipid lowering therapies can be instituted earlier in life.

We should also consider how genetic information can help us better understand healthy cardiovascular aging, which implies studies across a variety of population groups that includes men and women. For example, we understand some of the genetic differences related to age but race/ethnicity, but connecting these differences to metabolic underpinnings relevant to cardiovascular health and disease will be important areas of research in the future.

Discovery of novel risk factors and biomarkers for CVD is a very active field of investigation. Great hope has been expressed that we will develop personalized medical care strategies to appropriately diagnose, treat, and prevent CVD outcomes. Building on the concepts presented earlier, we need to recognize that methods to analyze data are in place or are being developed to help assess the utility of newer biomarkers. For example, it may be helpful to assess the added value of several factors that cluster together and principal components analysis may further the understanding of the factor being evaluated. Multiple measures of a factor over time may help to provide better estimates of risk than a single measurement, using methods that are analogous to integrating the area under a response curve that is used in metabolic experiments. Risk factors for outcomes in studies of longer duration may differ in comparisons with studies of shorter durations.

Clinical research concerning biomarkers and vascular disease outcomes has expanded greatly and there will continue to be a great deal of research in this arena. Table 3 lists some of the future directions for novel risk factor and biomarker investigation related to CVD. Population research will include further efforts to show incremental value, assessment of methods that provide for reclassification, and information concerning using biomarker data for persons to assess prognosis. Research related to integrating metabolism and genetics related to cardiovascular risk will include assessments of
the relative usefulness of gene information and biomarker information. It is likely that markers that affect risk and metabolism will be understood better with such research. Pollex and Hegele24 has suggested that “markers that cannot be measured in any way other than by genomic technologies might one day be added to diagnostic algorithms.” Investigating the determinants of small steps along the causal pathway atherosclerotic disease is also likely to rewarding in terms of understanding the pathophysiology of CVD. What about traditional risk factors? Many will continue to be useful in populations and for integrative science. Some will be replaced as the next generation of biomarker data are published. And yes, some risk factors have already mutated into genomics, proteomics, and metabolomics as cardiovascular research evolves.

Disclosures

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