Peripheral Arterial Disease

Genetics of Peripheral Artery Disease

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Broadly defined, peripheral vascular disease refers to disease of the extracardiac blood vessels, including diseases of the arteries, veins, and lymphatics. Peripheral arterial disease (PAD) refers to disease affecting noncoronary arteries, but is most often used to describe disease of the arteries supplying the limbs. Peripheral arterial disease is most commonly due to atherosclerosis, but may also be secondary to cardiac or vascular embolism, vasculitis, hypercoagulopathy, vascular dissection, vascular compression syndromes, and other less common disorders. In addition, PADs include those characterized by fixed or dynamic stenoses, and aneurysmal disease such as abdominal aortic aneurysm, as well. In this review, we focus on atherosclerotic arterial occlusive disease affecting the vessels supplying blood flow to the lower extremities (PAD), and discuss our current understanding and the future directions of PAD genetics.

PAD is a significant public health problem, and a major source of morbidity and mortality that affects ~8 million Americans. PAD contributes to impaired quality of life (eg, intermittent claudication reducing mobility), morbidity (eg, nonhealing ulcers and ischemic rest pain), and mortality (generally owing to its association with coronary and carotid artery disease). PAD is responsible for approximately half a million hospitalizations and 100 000 angiograms annually.1,2 In part, because of a general unfamiliarity with these diseases among the primary care community, PAD patients receive suboptimal treatment in comparison with patients with coronary artery disease (CAD), being prescribed therapeutic doses of statins, antihypertensive medicines, and antiplatelet agents less commonly than patients with CAD.3–6 Much remains unknown about the biological origins of this disease and how to effectively identify and treat affected individuals.

Using Genetics to Identify Pathophysiological Pathways and Novel Therapeutic Targets

A greater understanding of how genetic variation influences susceptibility to PAD may inform the development of novel therapeutics. High-throughput, whole-genome technology efforts have recently made inroads toward these goals (Table). The advent of genome-wide association studies (GWAS, discussed below) and cDNA microarrays (which measure the mRNA expression levels of all coding genes) has allowed for the unbiased detection of pathways that are differentially activated in affected versus unaffected individuals. Whereas these are the contemporary tools now driving genetic investigations of PAD, it is important to first put this recent work into a historical context.

Earlier Studies Documenting the Heritability of PAD

PAD is a complex disorder from a genetic standpoint. Unlike monogenic vascular syndromes such as Marfan and Loey Dietz that manifest a Mendelian inheritance pattern,14 atherosclerotic PAD likely results from dozens or hundreds of genes interacting with each other and the environment to cause disease.7 Epidemiological studies suggest that >50% of the population burden of PAD is attributable to classical risk factors such as smoking, diabetes mellitus, dyslipidemia, and hypertension.15 The remaining risk is thus accounted for by other unmeasured environmental or genetic components.

Several studies indicate a heritable component to PAD. In a study of patients with premature PAD (onset before age 50), half of the subjects’ asymptomatic siblings had occult lower-extremity atherosclerosis as determined by duplex ultrasonography.16 A recent Swedish twin study17 used discharge diagnosis to define PAD, and is likely more representative of patients with symptomatic claudication or critical limb ischemia. Genetic effects accounted for 58% (95% CI, 50%–64%) and nonshared environmental effects for 42% (95% CI, 36%–50%) of the phenotypic variance among twins in this report. Three studies conducted to date estimate that 21% of the interindividual variation in ankle-brachial index (ABI) is attributable to inherited factors. These studies include the National Heart, Lung, and Blood Institute (NHLBI) Twin Study,18 the Genetic Epidemiology Network Arteriopathy Study (GENOA),19 and a study performed in the Framingham Offspring Cohort.20 Many of the participants in these studies had ABI values in or near the reference range. Although sibling studies frequently overestimate attributable genetic risk because of shared environmental risk factors within families,21 these reports each provided evidence that ABI is at least moderately inheritable and justify the search for responsible gene variants. Historical methods included case-control approaches and linkage analyses.

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(Circulation. 2012;125:3220-3228.)

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.111.033878

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A family-based approach in which the genome is scanned for prespecified DNA markers that are known to be highly variable (ie, microsatellites). Regions that are found more commonly in the diseased members of the family are said to be linked to the causative gene, which is then pursued with fine mapping.

- Also known as positional cloning
- Requirements of previous knowledge of the causative genes
- Is particularly useful for single-gene, Mendelian disorders
- Can be executed with a relatively small number of well-pedigreed families
- Scans the entire genome

- Can only detect alleles with large effect sizes (ie, is not likely to be useful in multifactorial conditions such as PAD)

- Requires families with affected individuals across multiple generations (ie, is more difficult to perform in late-onset and highly morbid conditions such as PAD)

- Requires relatively large sample sizes that can be confounded by incomplete phenotyping of patients (ie, patients who have subclinical atherosclerosis may be erroneously identified as normals).
- Typically not powered to detect associations for rare variants (ie, polymorphisms with allele frequencies <1%)

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**GWAS indicates genome-wide association studies; PAD, peripheral arterial disease; PAOD, peripheral arterial occlusive disease; SNP, single-nucleotide polymorphism; CAD, coronary artery disease; MTHFR, methylene tetrahydrofolate reductase; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule 1; AAA, abdominal aortic aneurysm; and nACh, nicotinic acetylcholine.**

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**Earlier Studies to Identify Genetic Determinants of PAD**

**Candidate Gene Studies**

With use of the candidate gene approach, one searches for an association between a specific variant in a specific gene (eg, a single-nucleotide polymorphism, or SNP) and a clinical phenotype (generally defined by a low ABI in PAD patients). Such polymorphisms may alter a gene’s expression by altering the binding of its required transcription factors, impairing the stability or intracellular trafficking of its mRNA transcript, or limiting its ability to be translated into a functional protein. Often, the polymorphism may be linked to
another gene that is responsible for the disease. Collections of cases and controls are assembled and genotyped for variants in a suspected pathway. Driven by our understanding of atherosclerotic disease, most efforts thus far have focused on genes that are known or suspected to be related to modulating lipids, blood pressure, vasomotor tone, inflammation, or thrombosis. Association studies have included genes related to coagulation and platelet aggregation (prothrombin, Factor V Leiden), leukocyte activation (interleukin 6, E-Selectin, intercellular adhesion molecule), and endothelial function (nitric oxide synthase 3), among others (reviewed in 7,8,23).

Unfortunately, these studies have shed little light onto the pathophysiology of PAD. Plagued by small sample sizes and inadequate statistical power, many of the originally positive studies have not been successfully replicated in independent cohorts, suggesting that the original association was falsely positive. Concerns over adequate matching of racial/ethnic groups (who are known to have different rates of PAD, and different allele frequencies, as well) are justified by the possibility that “population stratification” may lead to spurious associations. Moreover, a significant proportion of the candidate gene studies reported to date do not conform to Hardy-Weinberg equilibrium (ie, the genotype was not distributed across the population as predicted by classical genetics) suggesting systematic genotyping errors or selection bias. Perhaps most concerning, a number of these studies appear to have been unblinded, additionally casting doubt on their conclusions.

Taken together, these candidate gene studies are inconclusive. Certain variants that have been identified appear promising and warrant additional investigation, such as polymorphisms in the homocysteine pathway regulating enzyme methylene tetrahydrofolate reductase,26–29 the inflammatory cytokine interleukin 6,30,31 and the vascular intercellular adhesion molecule.30,32 Finally, a major limitation of candidate gene studies is that they are not likely to uncover novel mechanisms, because the choice of gene variants is typically determined by previous observations indicating a putative role for the gene in atherosclerosis.

**Linkage Studies**

Family-based linkage studies have also been applied to understanding vascular diseases. In this approach, the genome is scanned at a low resolution (every 10 cM) for markers known as microsatellites that are coinherit with the phenotype of interest (typically several million base pairs between tags). Once a marker has been firmly associated across the population as predicted by classical genetics) suggesting systematic genotyping errors or selection bias. Finally, a major limitation of candidate gene studies is that they are not likely to uncover novel mechanisms, because the choice of gene variants is typically determined by previous observations indicating a putative role for the gene in atherosclerosis.

In the realm of PAD, however, the results have been somewhat disappointing. To date, only 1 positive linkage study has been reported. This study of 116 extended Icelandic families (884 subjects) identified a locus on chromosome 1p31 that was associated with angiographically or surgically documented PAD. This locus, known as PAOD1, had a logarithm of odds score of 3.93 (>3 is significant). Moreover, this locus was independent of other vascular risk factors, and the association was strengthened when stroke patients were removed. Together, these findings suggest that the associated gene may specifically predispose patients to vascular disease in the lower-extremity vascular bed. Unfortunately, the causative gene on chromosome 1 has not yet been identified, and this association has not been replicated by other investigators. The only other sizable linkage study performed to date, which utilized only ~1/3 the number of microsatellite makers studied in the preceding study, failed to definitively identify a significant genomic locus.

To be effective, linkage studies require extended family pedigrees and genes with large effect sizes. Because PAD tends to affect older adults, it is difficult to compile large collections of affected families. Furthermore, this disease is polygenic and results from a number of factors each with a modest effect on risk, rather than 1 dominant gene that will be easily detected. Together, it is not surprising that this approach has not met with more success in PAD.

**GWAS Studies**

Recent major advances in human genetics promise to overcome each of these deficits. In 2001, the Human Genome Project was completed, fully codifying the 3.1 billion nucleotides that make up our genetic code. Perhaps even more importantly, the International HapMap project provided a catalog of common polymorphisms across the genome in 2004, allowing us for the first time to study the natural variation that makes each individual unique. These tools, combined with technological advances that have enabled the relatively inexpensive genotyping of millions of tag SNPs simultaneously, have revolutionized modern genomics research. With the use of the GWAS approach, researchers can now scan the full genomes of large cohorts of patients to identify variants that are overrepresented in subjects with a given disease in comparison with unaffected controls (see review in 18,39). Unlike the candidate gene approach, the GWAS platform allows for an agnostic approach in which no prior knowledge is required to implicate novel biological pathways. Furthermore, the genome-wide approach allows for the consideration of polymorphisms in so-called gene deserts that do not encode any of the known ~20,000 protein-coding genes. These areas may contain noncoding elements that can modify gene expression, such as long noncoding RNAs.

Unlike conventional linkage analysis using microsatellites, the GWAS technology scans the genome at much higher resolution and with greater fidelity. Moreover, late-onset diseases with high mortality rates (such as PAD) can be studied without the need for extended family pedigrees, and genes with modest effect size (odds ratio of 1.2) can be detected. One drawback is that this approach requires large
numbers of subjects (thousands), and, because of multiple comparisons, the level of significance must be very high for a variant to be reliably associated with disease. Nevertheless, this approach has already provided revolutionary insights in several fields, as typified by the implication of the complement pathway in macular degeneration, autophagy in Crohn disease, and hedgehog signaling in human height.51,52

Since 2006, multiple loci have been definitively associated with cardiovascular disease.44 These lead SNPs have been replicated by several consortia in multiple racial ethnic groups. The strongest and most consistent association with cardiovascular disorders is with the intergenic portion of chromosome 9p21 \((P = 1.6 \times 10^{-7})\),11 which has been queried in well >100 000 patients.45,46 Variants at this locus have been reported to be responsible for as much as 21% of one’s lifetime risk of myocardial infarction.47 Importantly, these same polymorphisms also correlate with risk in the periphery, including aneurysmal disease, stroke, and arterial stiffness.12,48 The link to peripheral vascular disorders is most robust for abdominal aortic aneurysm and intracranial aneurysms (accounting for 26% of the attributable risk for these diseases), and persists even after removing patients with a history of myocardial infarction. A follow-up study on the representative 9p21 SNP rs1333049 supported this association with PAD status, and the severity of ABI in a cohort of older individuals (mean age 76), as well.49 The association persisted after controlling for diabetes mellitus, smoking, lipid levels, prior myocardial infarction, and hypertension, suggesting a novel pathophysiological mechanism at play.

The fact that the 9p21 SNPs correlate with disease status independently of known traditional risk factors has increased the enthusiasm for understanding the biology mediated by these SNPs. It is notable that these same SNPs also predict risk of the intracranial nonatherosclerotic berry aneurysm.12 Taken together, we and others have postulated that 9p21 likely does not alter vessel wall inflammation, thrombosis, or lipid accumulation, but rather it regulates the structural integrity of the artery itself. Given the particularly strong link to aneurysmal disorders, it is likely that the dominant role of 9p21 variants in vivo will center around vascular smooth muscle cell physiology and cell-fate decision making.50

Although much work has focused on the 9p21 hits described above, it is worth highlighting that a number of other GWAS polymorphisms have been implicated as significant at the genome-wide level. Because the majority of these localize to genes not previously implicated in vascular disease, they certainly warrant investigation and should also yield novel biological targets. For example, one of the most exciting leads comes from a recent GWAS that found an associated SNP within a cluster of genes that encode nicotinic acetylcholine receptors.13 This variant not only correlated with PAD (10% of the attributable risk for disease), but also predicted nicotine dependence and number of cigarettes smoked per day. It may be that the link to PAD occurs indirectly (ie, by increasing lifetime smoking burden) or possibly by directly modulating the effect of tobacco on the vasculature. In this regard, we have shown that nicotine can directly accelerate plaque neovascularization and atherosclerosis by stimulating vascular nicotinic acetylcholine receptors.51,52

Whereas the genetic variations discovered by GWAS have been predictive of CAD and PAD, as well, it is likely that genetic variations that are more specific for PAD ultimately will be uncovered. Indeed, it is already apparent that the conventional risk factors have different predictive value for PAD, with tobacco exposure and diabetes mellitus being stronger risk factors for PAD than is dyslipidemia.53,54 Consistent with these differences in pathophysiological determinants is the observation of a different proteomic profile in patients with PAD and CAD (PAD/CAD), than those patients with CAD alone. Specifically, \(\beta_2\)-microglobulin, cystatin C, and C-reactive protein are each found in higher levels in PAD/CAD and can be used together to stratify the risk of PAD in a susceptible population.55,56

Use of Genetics in PAD Detection and Treatment

A greater understanding of the genetic underpinnings of PAD could enhance our capacity to detect those at risk for disease. PAD is underdiagnosed, and these patients are not receiving medication known to reduce morbidity and mortality. Only 10% to 30% of PAD patients manifest the classic symptom of intermittent claudication.3,57 Although the ABI test, which measures the ratio of blood pressure in the lower and upper extremities, is a simple and useful office-based technique to detect PAD,58 evidence indicates that this test is underutilized. In fact, the Peripheral Arterial Disease Detection, Awareness, and Treatment in Primary Care (PARTNERS) ABI screening study of nearly 7000 adult general medicine patients found that over half of those with PAD had been previously undetected.3 Even when widely implemented, the ABI does not correlate with functional status and only poorly with disease progression.59,60 A blood test that could identify those at greatest risk for PAD could focus additional screening toward those populations.

Currently, genetic screening to assess the risk or progression of PAD is far from being realized. However, the possibility of such screening in the future is foreshadowed by recent advances in cardiac transplantation. In cardiac transplant recipients, the AlloMap test (which measures the expression of 11 genes in peripheral blood samples) has been shown to accurately predict allograft rejection.61 This blood test has the potential of replacing the current screening methodology that is invasive and expensive (ie, frequent endomyocardial biopsies and echocardiograms). Clearly, standardized, off-the-shelf blood tests that will obviate the need for technical imaging studies would greatly enhance our ability to rapidly identify at-risk individuals, initiate therapies early in the course of disease, and prognosticate with greater accuracy. Given the fact that as many as 60 million Americans technically meet the guideline criteria for a screening ABI, it is clear that an effective PAD panel could focus healthcare resources on those most at risk.55

More powerful tools, which will arise from our forthcoming ability to rapidly and cheaply sequence the entire genome, will certainly provide further insights, point out additional targets, and allow for therapies tailored to an individual’s personal genetic makeup. To this final point, the field of pharmacogenomics, which studies the role of genetic differ-
ences in the response to drug therapy, has been rapidly expanding. A clear example relevant to PAD comes from the discovery that carriers of the common loss-of-function cytochrome p-450 enzyme polymorphism, CYP2C19, metabolize clopidogrel (the most efficacious antiplatelet drug in PAD) significantly more slowly than unaffected controls (reviewed in86). These patients have reduced platelet inhibition on standard clopidogrel doses and experience a higher risk of major adverse cardiovascular events. As such, the Food and Drug Administration has provided a black-box warning for this agent in so-called poor-metabolizers. We do not yet know if higher doses of clopidogrel will reduce the risk of future event (without increasing bleeding). However, it is clear that clinicians will soon need to become facile with the concept of pharmacogenomics when choosing a drug for a particular patient. Before long, we anticipate that a patient’s genetic variations will be documented and will factor into drug selection similarly to other current considerations, such as age, weight, and concurrent medications.

The Future of PAD Genetics

Gene-by-Environment Interactions

Gene-by-environment interactions are hypothesized to play an important role in the expression of disease, and have spurred investigations into the pathophysiological synergy of genomic and environmental exposures. Recently, this topic was approached with the first environment-wide association study that identified the pesticide heptachlor epoxide as being associated with type II diabetes. Application of similar environment-wide scans will likely also be useful in PAD.

Epigenetic Factors

Additionally, we will become more sophisticated in our evaluation of noncoding RNAs and other epigenetic modifications that promote PAD. The microRNAs are diffusible ≈22 nucleotide single-stranded RNAs that target coding mRNA transcripts for degradation. Although discovered less than a decade ago, they are now known to regulate upward of 50% of the entire genome. This remarkable pathway, which consists of only ≈1000 genes, has already been implicated in a number of pathways relevant to PAD including impaired diabetic neoangiogenesis, smooth muscle remodeling, and circulating endothelial progenitor cell number. The advent of microarrays that can analyze the small RNA subfraction will certainly identify other microRNAs that are relevant to peripheral arterial disease.

Beyond microRNAs, tools now also exist to measure other epigenetic factors in PAD, such as histone modification, chromatin remodeling, and DNA methylation. These processes induce structural modifications to the DNA molecule itself, rather than alterations in the DNA sequence. Changes in DNA methylation or chromatin modifications make a given gene more or less amenable to transcription, and thus can alter the expression of that gene. Epigenetic alterations may occur postnatally and reproducibly localize to certain regions of the chromosome in disease. Detecting these patterns can provide insights that will implicate causative genes. It is possible that epigenetic signatures can even be inherited across families and that traits acquired because of environmental exposure can be passed to offspring to contribute to the familial concentration of disease. Although most of the cardiovascular epigenetic research completed to date has focused on pathways related to atherosclerosis and vascular biology, in general, interesting studies are forthcoming in abdominal aortic aneurysm disease and should also prove informative in PAD.

Acquired Mitochondrial Genetic Alterations

There is accumulating evidence for a mitochondriopathy in PAD that contributes to the impairment in functional capacity. The mitochondriopathy may be due in part to acquired alterations in mitochondrial DNA (mtDNA). In the patient with PAD, intermittent claudication is associated with ischemia-reperfusion, a known stimulus for generation of reactive oxygen species. Regular bouts of ischemia-reperfusion may damage mtDNA, which is particularly vulnerable because of its proximity to reactive oxygen species generated by the electron transport chain, and because of its lack of protective histones. Indeed, in comparison with limbs of age-matched subjects without PAD, the skeletal muscle mitochondria in the most affected limb of patients with PAD have almost 20-fold greater frequency of the 4977-bp mtDNA deletion. These mitochondrial genetic abnormalities may explain in part the mitochondriopathy of PAD. Notably, the only electron transport chain protein activity that seems unaffected in PAD skeletal muscle is that of complex II, which is encoded by nuclear, rather than mitochondrial DNA. However, similar mtDNA alterations can be seen in the unaffected limb in patients with unilateral PAD, suggesting that systemic oxidative stress or other factors may be contributing to the mtDNA abnormalities in PAD. In any event, medical therapy to reduce mitochondrial injury or enhance mitochondrial function may represent an interesting therapeutic avenue in PAD.

Comparative Genomics

Known differences in susceptibility to hindlimb ischemia between mouse strains are also now being exploited to advance our understanding of PAD and angiogenesis. Using an elegant comparative genomics approach, Dokun and colleagues performed a linkage analysis on mice from 6 genetic backgrounds that had undergone femoral artery ligation. They identified a quantitative trait locus on chromosome 7 that influences wound healing in situations of tissue hypoxia and critical limb ischemia. Other investigators have found that this same locus also appears to control >50% of the variability in infarct burden in a mouse model of ischemic stroke. Although the molecular mechanisms downstream of this region have yet to be defined, this example highlights the power of leveraging the natural differences that occur because of genetic variation between inbred animal lineages.

Exomic and Whole Genome Sequencing

The most important advances, however, are likely to occur to as a result of the even more powerful genetic tools on the horizon. Although the GWAS platform has provided new insights that will reshape our approach to PAD genetics, this tool is not without limitations. High-density genotyping
chips now can identify up to 2.5 million individual markers per subject, but they rely on imputation to predict the remaining SNPs in our genome. Rare, personal variants (ie, occurring with a frequency of <1% across the population) are not accurately cataloged for consideration with the GWAS approach. Moreover, historical GWAS genotyping has had little if any power to detect so-called structural variants such as insertions, deletions, and copy number variants, all of which have been implicated in vascular disease. The advent of exome and truly full genome sequencing, where all existing nucleotides are codified, will address these limitations. Companies such as Complete Genomics and Illumina now provide full genome sequencing services for as little as a few thousand dollars per patient (depending on order size82) and can provide this information in a matter of days (versus the $3 billion and 10 years required to sequence the first genome in the Human Genome Project). Because Moore’s Law on the declining cost of computing continues to be outstripped by the falling price of sequencing, it will not be long until standard academic laboratories will have bench-top sequencers capable of scanning entire genomes for ≤$1000 per genome.83

PAD Collaborations and Advanced Informatics

Most importantly, this generation will see the creation of larger and more successful collaborations focused on the investigation of PAD. With superior phenotypic characterization and longer-term follow-up, these international groups will organize studies specifically intended for combining datasets and optimizing future meta-analyses.23 Novel approaches will focus on collecting genetically enriched subjects to examine the extremes of phenotypes. For example, our group has collected subjects with very early onset of atherosclerotic disease (<45 years of age) in the ADVANCE study,84 and others have chosen to study the opposite end of the spectrum with subjects >80 of age who have no medical comorbidities (the Wellderly).

In addition to the creation of consortia for conducting genetic studies of complex diseases such as PAD, there is considerable interest in leveraging the electronic medical record for high-throughput phenotyping to facilitate such studies. The Electronic Medical Records and Genomics (eMERGE) network (www.gwas.org) was established in 2007 to develop and implement methods for leveraging biorepositories linked to electronic medical record systems for large-scale genomic research.85,86 One of the participating sites in this network, Mayo Clinic, is conducting a GWAS of PAD that includes 1648 cases and 1675 controls87 recruited in the clinical setting and with linkage to the electronic medical record. Mining of structured data from the electronic medical record, and natural language processing of unstructured text, as well, were used to obtain relevant covariates.87

Conclusions

PAD is an important and highly prevalent condition with a heritable component. Vascular biologists and geneticists have begun to make inroads on this complex disease by applying increasingly sophisticated genetic approaches (Figure). We look forward to the next decade of research, as evolving technologies and interdisciplinary collaborations promise to finally provide insights on this often morbid condition.

Sources of Funding

This work was supported by grants from the National Institutes of Health (K12HL087746 and U01HL100397 to Dr Cooke and U0–1 HG-04599 to I.J.K.) and the American Heart Association (10BGIA3290011 to Dr Leeper).

Disclosures

None.

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Key Words: peripheral artery disease • genome-wide association studies • genomics
Genetics of Peripheral Artery Disease
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Circulation. 2012;125:3220-3228
doi: 10.1161/CIRCULATIONAHA.111.033878
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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