Genetics of Ultrasonographic Carotid Atherosclerosis

Teri A. Manolio, Eric Boerwinkle, Christopher J. O’Donnell, Alexander F. Wilson

Abstract—The search for genes related to the cause of common complex disorders such as cardiovascular disease has been frustrating, partly because of the many factors known to contribute to cardiovascular disease and the potential “distance” of cardiovascular disease as a phenotype from genes and gene products. Linkage and association studies for phenotypes more proximal in the pathway from DNA sequence variation to overt clinical disease, such as ultrasound-defined carotid atherosclerosis, may potentially be more enlightening. Only one genetic variant previously reported to be associated with atherosclerosis or clinically evident cardiovascular disease, matrix metalloproteinase (MMP) 3, has shown consistently positive associations with carotid disease, although it has not been studied widely. Another, PON1 L55M, is weakly associated in subgroups only, and 2, ApoE and MTHFR, are equivocal. Genetic variants reported to be associated with clinical cardiovascular disease show weak or no relationship to carotid atherosclerosis. This may reflect the known inconsistency in associations of genetic variants with clinical cardiovascular disease itself. Alternatively, genetic determinants of ultrasound-defined carotid atherosclerosis may differ from those of clinically manifest cardiovascular disease and may require pursuit through large-scale genomic studies of carotid atherosclerosis as a distinct phenotype. (Arterioscler Thromb Vasc Biol. 2004;24:1-10.)

Key Words: atherosclerosis ■ genes ■ human ■ carotid artery ■ cardiovascular disease
near wall as the far wall, however, and atherosclerosis progression rates are similar, with the most precise measure probably being combined near and far wall thickness.\textsuperscript{11} Reported differences in relationships of risk factors and disease incidence with IMT measured at different carotid sites have raised the possibility of site-specific differences in their cause and, possibly, their genetic determinants.\textsuperscript{12} Development and progression of atherosclerotic lesions of the internal carotid, where flow is turbulent, have been suggested to be related primarily to lipid accumulation and plaque hemorrhage, whereas the laminar flow typical of the common carotid may lead to more diffuse medial thickening indistinguishable ultrasonographically from atherosclerosis.\textsuperscript{3,12,13} This may explain why internal carotid IMT has been more strongly related to early parental history of stroke, whereas common carotid IMT may be more strongly related to early parental history of myocardial infarction.\textsuperscript{10}

The metric commonly used to summarize the familial and genetic nature of a trait is heritability (H, h$^2$, or $\sigma^2_h/\sigma^2$). The heritability of a trait is the proportion of interindividual variation in the trait ($\sigma^2_h$ attributable to genetic variation ($\sigma^2_g$). Heritability of a trait is a population- and environment-specific parameter, and its value, high or low, does not indicate the role of genes in any specific individual or patient. Heritability does, however, allow one to predict the expected degree of familial aggregation of a trait, and traits with a high heritability should prove fruitful in identifying trait-related genes.

One of the first formal assessments of heritability of carotid atherosclerosis was reported for carotid IMT in 46 sibships in Mexico City.\textsuperscript{21} The estimate of heritability was high, 92\%, for the common carotid, and 82\% for the internal carotid, after adjustment for standard CHD risk factors (Table 1). These high estimates have been questioned and subsequent reports have consistently estimated heritabilities in the range of 20\% to 40\% in unselected subjects,\textsuperscript{22,23} twins,\textsuperscript{24,25} and subjects with type II diabetes,\textsuperscript{26} although they are somewhat higher in families ascertained through a hypertensive parent\textsuperscript{27} and in randomly ascertained families.\textsuperscript{12}

### Table 1. Heritability of Various Carotid Artery Phenotypes.

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CVD indicates cardiovascular disease.
the range of 0.75 mm to 1.5 mm; \(^{24,30–35}\) and focal acceleration of flow as measured by Doppler spectral analysis.\(^ {7,36}\) Reference may also be made to “carotid atherosclerosis,” also typically defined as thickening greater than a given threshold, whether focal or not.\(^ {37,38}\) A plaque score or “B” score developed for the Asymptomatic Carotid Artery Plaque Study\(^ {39}\) based on increasing categories of IMT has also been used,\(^ {40–42}\) whereas other studies have calculated plaque scores by summing the maximal thickness of each plaque in the carotid arteries bilaterally.\(^ {43}\)

Plaque as a carotid atherosclerosis phenotype is not studied as frequently as carotid IMT. The heritability of plaque determined by localized IMT \(\approx 1.5\) mm was recently estimated at 23% to 28%.\(^ {35}\) Carotid plaque has also been reported to be more strongly related to early parental CHD death than is IMT.\(^ {44}\) Whereas one would anticipate that genes influencing plaque overlap with those influencing IMT, there are likely to be unique sets of genes related to both.\(^ {45}\)

Other Carotid Phenotypes
Other phenotypes related to carotid atherosclerosis include plaque echogenicity (consistent pathologically with more organized and fibrotic plaques) or echolucency (consistent with greater lipid deposition, intraplaque hemorrhage, and vulnerability to rupture),\(^ {46}\) lumen diameter, distensibility, and stiffness. Lumen diameter is not widely reported because it correlates relatively poorly with atherosclerosis in its early stages, probably because the vessel dilates to preserve the lumen from encroachment until fairly late in the disease.\(^ {10}\) Distensibility and stiffness are related to the elasticity measures described\(^ {14}\) and are often calculated as the difference between minimum and maximum luminal diameter during the cardiac cycle multiplied by some index of blood pressure.\(^ {47}\) Few reports of heritability of these measures are available, although one study did demonstrate higher heritability of lumen diameter (0.44) than IMT (0.21) and similar heritability of arterial stiffness (0.23).\(^ {22}\)

Genetic Variants Related to Carotid Phenotypes
Several genetic variants have been examined in relation to carotid atherosclerosis, more commonly by association than linkage analysis. Most have related genetic variants to IMT as a continuous trait, although many have reported dichotomous carotid plaque measures derived from arbitrary threshold levels of IMT as described. Findings from the most widely studied variants are summarized, followed by a listing of variants only beginning to be explored in relation to carotid disease.

Angiotensin 1-Converting Enzyme
Perhaps the most studied locus in relation to carotid atherosclerosis, and cardiovascular disease in general, is the insertion/deletion polymorphism of the angiotensin-converting enzyme (ACE) gene.\(^ {48}\) Presence (insertion, I) or absence (deletion, D) of a 287-bp alu-repeat sequence in reverse orientation in intron 16 of this gene is associated with substantially different levels of plasma ACE activity in a codominant fashion, with DD homozygotes having the highest levels.\(^ {48}\) ACE converts inactive angiotensin I to the vasoconstrictor angiotensin II and also inactivates the vasodilator bradykinin, leading to increased vascular tone, vascular smooth muscle cell growth, neointimal proliferation, and extracellular matrix deposition.\(^ {34,49}\) Variants associated with higher ACE activity might thus be expected to be related to increased carotid wall thickness and plaque formation.

Evidence for such an association is inconsistent, however, with the majority of studies showing no association\(^ {23,31,33,46–49}\) whereas several show higher IMT and plaque frequency in DD homozygotes or D allele carriers\(^ {34,36,63–73}\) (Table I, available online at http://atvb.ahajournals.org). In only one was higher IMT associated with the II genotype, but this was in a small number of patients with symptomatic carotid territory cerebral ischemia.\(^ {64}\) Most studies of the ACE genotype and carotid atherosclerosis, regardless of findings, include \(<500\) subjects, with the 2 largest (\(\geq 1000\)) and \(4000\) subjects) showing no association. Only 2 studies reported possible interactions of the D allele with other factors in relation to IMT, in that IMT increased more steeply with increasing systolic blood pressure\(^ {61}\) and age\(^ {61}\) in D allele carriers than in II homozygotes.\(^ {61}\) Another showed an association of the DD genotype with echolucent (high-risk) plaques despite lack of association with IMT or stenosis.\(^ {46}\)

Apolipoprotein E
Another widely studied gene in relation to carotid atherosclerosis is apolipoprotein E (APOE), whose role in cardiovascular disease has been reviewed by Eichner et al.\(^ {74}\) Three common alleles (designated e2, e3, and e4) produce 3 protein isoforms differing at amino acid positions 112 and 158. The most common allele, APOE*e3, produces the Apo E3 isoform with cysteine at position 112 and arginine at 158, whereas the least common, APOE*e2, produces the Apo E2 protein with cysteine at both positions, and Apo E4 has arginine at both positions. Apolipoprotein levels vary by polymorphism, with APOE*e2 associated with higher and APOE*e4 associated with lower plasma apo E levels.\(^ {75}\) Apo E2 in turn is associated with \textit{lower} low-density lipoprotein
(LDL) cholesterol levels, and Apo E4 with higher levels, than is the Apo E3 isof orm.76 Carrying an APOE*e4 allele has generally been associated with modestly increased risk of coronary heart disease (and strongly increased risk of Alzheimer disease), and carrying an APOE*e2 allele has been associated with lower risk, compared with the e3/e3 genotype,75 although the APOE*e2 allele has also been related to increased risk.74,77

Most studies of the APOE e2/e3/e4 polymorphism have shown carotid IMT to be lower in carriers of the e2 allele and higher in carriers of the e4 allele, compared with e3/e3 homzygotes76,78–84 (Table II, available online at http://atvb.ahajournals.org). These associations have not been totally consistent, however, with de Andrade58 and Hanon55 showing higher IMT in e2 carriers, Zannad52 showing higher IMT in e3/e4 homzygotes compared with e2 or e4 carriers but on the right side only, and several studies30,37,49,56,62,68–86 showing no association. Both studies reporting higher IMT in e2 carriers excluded persons with prevalent cardiovascular disease, which excludes proportionately more persons with carotid thickening,78 although relationships of APOE and IMT have been suggested to be similar regardless of prevalent disease.78 Most positive findings have remained after adjustment for multiple cardiovascular disease (CVD) risk factors and particularly for lipoprotein levels, through which the apo E polymorphism might be expected to exert some of its effect.75 Associations in diabetics68,80 and dialysis patients56,79 have tended to be less consistent than those in unselected populations. Interactions with environmental or context-dependent factors have been infrequently examined, although 2 studies suggested an interaction of the e4 allele with smoking to produce increased carotid atherosclerosis,57,58 and 1 formally tested and demonstrated a greater increase in IMT with increasing body mass index in e4 carriers.58 Another demonstrated a greater increase in plaque frequency per “unit change” in genotype in women than in men.84

Angiotensinogen and Angiotensin II

Type 1 Receptor

Polymorphisms in the genes for angiotensinogen (AGT) and angiotensin II type 1 receptor (AGTR1) have been associated with increased cardiovascular disease risk.90,91 AGT is the precursor peptide of angiotensin II, a potent vasoconstrictor precursor peptide of angiotensin II, a potent vasoconstrictor

together to have the highest carotid IMT.56

substitution at position 174 (T174M), also in exon 2. Both the T allele of the M235T variant and M allele of the T174M variant have been associated with increased angiotensinogen levels, hypertension, and/or coronary disease.96,97 A second polymorphism in the promoter region, an adenine for cytosine substitution (A-20C), is also in nearly complete linkage disequilibrium with the G-6A polymorphism and has been related to increased plasma AGT levels and risk of hypertension. Lastly, an A-to-C transversion in the AGTR1 at nucleotide 1166 (AGTR1 A1166C) in the 3’ untranslated region has been associated with increased risk of essential hypertension and higher prevalence of coronary disease.92

Studies of the AGT M235T polymorphism have fairly consistently shown no association with IMT in a variety of population samples56,58,73–100 although 1 study of 98 previously untreated hypertensive patients showed higher IMT at entry and greater reduction in IMT after treatment in TT homozygotes101 (Table III, available online at http://atvb.ahajournals.org). Losito et al71 also examined the T174M polymorphism, with no association demonstrated. Similarly, no associations were detected with the AGTR1 A1166C polymorphism.52,56,58,73,92,100,102 Only one study to date has examined the promoter region polymorphisms, with no overall association detected, but women carrying the AGT-6A and AGT-20C alleles were shown to have higher IMT after adjustment.92 Only 1 possible gene–environment interaction was reported, for the AGT M235T polymorphism, with TT homozygotes shown to have a steeper increase in IMT with increasing systolic blood pressure compared with M allele carriers.58 Two studies examined possible gene–gene interactions, with one suggesting that the ACE I allele is associated with increased IMT in AGT G-6A GG homzygotes only,92 and another showing carriers of the ACE D allele, AGT M235T T allele, and AGTR1 A1166C C allele together to have the highest carotid IMT.56

Methylene Tetrahydrofolate Reductase

Genetic variation in the enzyme methylene tetrahydrofolate reductase has been associated with increased levels of homocysteine and increased risk of coronary disease. methylene tetrahydrofolate reductase reduces 5,10-methylenetetrahydrofolate to produce 5-methyltetrahydrofolate, which acts as a carbon donor in the conversion of homocysteine to methio-
of T alleles, others showed increased atherosclerosis only in those without cardiovascular disease, or in men only, or in women only. This last analysis also suggested an interaction with cigarette smoking and alcohol consumption in that TT homozygous women who smoked or consumed alcohol had higher IMTs than those who did not; this difference was not seen for the other genotypes.

**Paraoxonase**

Three paraoxonase genes (PON 1, PON 2, PON 3) have been mapped to chromosome 7 and a number of polymorphisms have been identified. PON prevents lipid peroxidation of LDLs and PON 1 has been shown to hydrolyze lipid peroxides in human atherosclerotic lesions. Oxidative modification of LDL cholesterol renders it particularly atherogenic, making identification of factors protecting against oxidative modification potentially important in the prevention of atherosclerosis. Two frequent PON 1 polymorphisms at positions 192 (glutamine to arginine) and 55 (methionine to leucine) are in linkage disequilibrium and are associated with variations in PON activity to different substrates. Associations between PON 1 and PON 2 polymorphisms and a variety of CVD phenotypes, however, have been inconsistent, with some studies showing the 55L allele and/or the 192R allele to be associated with increased risk and others showing no association. Both cigarette smoking and diabetes are associated with increased lipid peroxidation, leading to examination of associations with CVD in subgroups defined by these traits, which have also been inconsistent.

Of 13 studies considering the PON 1 192 polymorphism separately or jointly with PON 1 55 (Table V, available online at http://atvb.ahajournals.org), 10 showed no association with PON 1 192 considered alone, although some studies showing the 55L allele and/or the 192R allele to be associated with increased risk and others showing no association. Both cigarette smoking and diabetes are associated with increased lipid peroxidation, leading to examination of associations with CVD in subgroups defined by these traits, which have also been inconsistent.

No association of the G894T variant with carotid atherosclerosis was found in 4 studies, although a fifth demonstrated T homozygosity to be more frequent in subjects who were homozygous for the PON 1 55 L and PON 1 192Q alleles compared with LL/RR and MM/QQ subjects, and another showed the PON 1 192R genotype to be more common than QQ in stenosis cases but only after adjustment for PON activity level. This report was subsequently refuted in an expanded sample from the same authors, who found no association regardless of adjustment for PON activity. An eleventh study showed higher IMT in RR homozygotes than Q allele carriers; the twelfth showed plaque to be more frequent in PON 1 192R carriers with high levels of high-density lipoprotein, although no difference in plaque by genotype in subjects with low levels of high-density lipoprotein. The thirteenth showed the R allele to be more frequent in older persons with moderate versus no atherosclerosis.

Association studies of the PON 1 55 polymorphism have suggested that IMT and plaque are increased in L allele carriers, but relationships are complex. Of 5 studies examining the PON 1 55 allele, 1 showed plaque score to be higher in LL homozygotes, 1 showed the L allele to be more frequent in stenosis cases but only after adjustment for PON activity level, another showed the aforementioned higher IMT in LL/QQ subjects, a fourth showed higher IMT in nonsmoking LL homozygotes but no association in smokers, and the fifth showed plaque to be more frequent in L allele carriers but only in nonsmokers, with a reversal of this relationship in smokers. Subsequent expansion of the sample showing increased L allele frequency in stenosis cases after adjustment for PON activity failed to confirm this association. Only 2 studies examined the PON 2 S311C polymorphism and found no relationship with carotid atherosclerosis. Examination of 2 additional promoter polymorphisms, as well as a haplotype comprising polymorphisms at −162/−108/55/192, failed to detect any association with stenosis cases.

**Nitric Oxide Synthase**

Nitric oxide is a potent vasodilator produced by the action of nitric oxide synthase (NOS) on L-arginine. NO also inhibits platelet aggregation and adhesion, leukocyte chemotaxis and adhesion, adhesion molecule and chemokine expression, smooth muscle cell proliferation and migration, and LDL oxidation. For these reasons, endothelial NO may have a prominent role in protection against atherosclerosis. NOS exists in 3 forms: endothelial, neuronal, and inducible. Endothelial NOS (eNOS or NOS 3) is presumed responsible for most of the endothelial and vascular effects of NO. Several polymorphisms of the NOS 3 gene have been identified, one of which, a G-to-T transversion at nucleotide 894 of exon 7, produces a glutamic acid to aspartic acid substitution at amino acid 298. This induces a conformational change that may reduce NOS 3 activity. This variant has been associated with enhanced vasoconstrictive response to phenylephrine; with enhanced blood pressure response to endurance training; and with hypertension, coronary disease, and myocardial infarction. A second polymorphism in the 5' flanking region (T-786C) reduces NOS 3 gene promoter activity and has been associated with coronary spasm. Multiple intronic polymorphisms of unknown significance have also been identified.

No association of the G894T variant with carotid atherosclerosis was found in 4 studies, although a fifth demonstrated T homozygosity to be more frequent in subjects with plaque, and another showed the T-786C polymorphism to be more frequent in stenosis cases but only after adjustment for PON activity level. No study of epistasis in hemodialysis patients reported increased plaque frequency in subjects with the T-786C polymorphism in combination with APOE*4; the same interaction was found for a NOS 3 intron 4 polymorphism tightly linked to the T-786C locus.

**Genes Related to Lipid and Lipoprotein Levels**

Multiple genes in pathways of production or metabolism of lipids and lipoproteins have been examined in relation to carotid atherosclerosis. (Table VII, available online at http://atvb.ahajournals.org). IMT is consistently higher and plaque more frequent in familial hypercholesterolemia patients, but specific mutations have not consistently been implicated. Associations with other mutations have either been inconsistent, not yet replicated (that is, single reports), or not detected.
Apolipoprotein E 19q13.32;50.10 Mb 8 with polymorphism
Angiotensin-converting enzyme I/D Framingham Heart Study.134 associations was replicated in a large sample from the Paraoxonase 1 Q192R 7q21.3;94.54 Mb 3 with R allele 10 No association Methylene tetrahydrofolate reductase Angiotensin II receptor type 1 A1166C 3q24;149.74 Mb 0 7 No association Angiotensinogen M235T 1q42.2;227.87 Mb 0 8 No association Nitric oxide synthase G894T 7q36.1;150.08 Mb 1 with T allele 4 No association Paraoxonase 1 L55M 7q21.3;94.54 Mb 5 with L allele, although many limited to subgroups
IL-6 G-174C 7p15.3;22.51 Mb 1 with G allele 3 No association

Other Polymorphisms
Genetic variants related to hemostatic and inflammatory factors, interleukins and immune response, platelet receptors, and oxidative pathways, have also been studied sporadically (Table VIII, available online at http://atvb.ahajournals.org). Associations with hemostasis-related variants have generally been absent132–134 or present only in subgroups.28,100 The Marburg I variant of factor VII activating protease and factor V Leiden, however, have been shown to be more frequent in individuals with plaque progression,135 whereas the V Leiden, however, have been shown to be more frequent in individuals with plaque progression.135 whereas the β-fibrinogen C148T polymorphism in the homozygous TT form was associated with higher plaque score,135 although none of these associations was replicated in a large sample from the Framingham Heart Study.134

Of the other variants studied, the 6A allele of the MMP3 5A/6A promoter polymorphism has been associated with higher IMT and stenosis in all 4 studies examining it.136–139 Four studies assessed the IL-6 G-174C polymorphisms but with inconsistent or negative results.137,139–141 Two studies examined the Leu7Pro variant in pre-pro-neuropeptide Y and both showed higher IMT in 7Pro allele carriers.142,143 Scattered reports of other polymorphisms are difficult to interpret but are included for completeness.

Summary
A wide variety of genetic variants previously reported to be associated with atherosclerosis or clinically evident cardiovascular disease has been examined for associations with carotid atherosclerosis. Only 1 of these, MMP3, has been consistently positive (although not widely studied); 1 (PON1 L55M) has weak associations in subgroups, and 2 (ApoE and methylene tetrahydrofolate reductase) are equivocal (Table 2).

Several factors may account for the discordance among these studies, including: (1) sampling error or random type I errors in positive studies; (2) lack of power in negative studies; (3) genetic heterogeneity; (4) population stratification or confounding; (5) gene–environment interactions modulating expression of an associated genotype; or (6) differences in measurement methods and reproducibility across studies.

Despite its high heritability, high measurement precision, and strong relation to subsequent CVD, all of which making ultrasonographic carotid atherosclerosis an attractive intermediate phenotype, genetic variants reported to be associated with clinical cardiovascular disease show weak or no relationship to carotid atherosclerosis. This may reflect inconsistency in associations with clinical cardiovascular disease itself, as many initial reports of candidate gene associations are not replicated in further investigation.144–146 An alternative hypothesis suitable for investigation is that genetic determinants of ultrasound-defined carotid atherosclerosis differ from those of overt cardiovascular disease. Because of its association with multiple cardiovascular diseases, discovery of the gene(s) for carotid atherosclerosis should be pursued through large-scale genomic studies as distinct studies unto themselves. In addition, sequence variants may not adequately capture the totality of heritable variation in this or other traits; epigenetic and posttranslational protein modifications should also be investigated for relationships to carotid atherosclerosis.

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