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Noninvasive Phenotypes of Atherosclerosis: Similar Windows but Different Views

J. David Spence
Robarts Research Institute, jdspence@uwo.ca

Robert A. Hegele
Robarts Research Institute

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J. David Spence, MD; Robert A. Hegele, MD

Background and Purpose—Noninvasive measures of atherosclerosis, such as carotid intima-media thickness, total carotid plaque area, and carotid stenosis, probably represent different phenotypes with distinct determinants. For instance, total carotid plaque area may reflect atherosclerotic lesion size more closely than carotid stenosis, which instead may reflect hemodynamic compromise within the arterial lumen.

Methods—In 1821 patients from a Premature Atherosclerosis Clinic, we studied determinants of total carotid plaque area and carotid stenosis as measured by ultrasound using multivariate regression analysis with traditional risk factors and some emerging risk factors.

Results—Regression modeling showed that (1) traditional atherosclerosis risk factors were more strongly associated with total carotid plaque area than with carotid stenosis ($R=0.53$ and 0.13, respectively), and (2) individual risk factors had different relationships with total carotid plaque area and carotid stenosis. For instance, age accounted for 53% and 26% of the explained variance of total carotid plaque area and carotid stenosis, respectively. Female sex was inversely associated with total carotid plaque area but positively associated with carotid stenosis. Nontraditional risk variables such as plasma homocysteine had different associations with the 2 analytes.

Conclusions—Total carotid plaque area and carotid stenosis had different associations with specific atherosclerosis risk factors. Thus, for future studies of the determinants of atherosclerosis, it is important to distinguish between different phenotypes and to appreciate that they will not necessarily have the same determinants. **(*Stroke*. 2004;35:649-653.)**

Key Words: atherosclerosis ■ carotid artery plaque ■ carotid stenosis ■ genetics ■ phenotype ■ risk factors

Studies of atherosclerosis encompass a broad range of phenotypes, including clinical events such as stroke or myocardial infarction, transient ischemic attacks or unstable coronary syndromes, and measurements derived from noninvasive assays with the use of lumenography, ultrasound, CT, or MRI. Noninvasive modalities can also measure different aspects of atherogenesis. For instance, ultrasound examination of the carotid arteries can provide determinations of intima-media thickness (IMT), total cross-sectional area of carotid plaques, or severity of arterial stenosis. Although these phenotypes each assay "atherosclerosis," they represent different stages of atherogenesis, which is a complex multistep process that has many physical, biochemical, molecular, and genetic determinants.[1]

Among carotid ultrasound determinations, IMT probably reflects a hypertrophic response of arterial intimal and medial cells to lipid infiltration[2,3] or hypertension.[4–6] In contrast, formed arterial plaques probably represent a later stage of atherogenesis related to inflammation, oxidation, endothelial dysfunction, and/or smooth muscle cell proliferation.[1] As plaques develop, there may be some compensatory arterial enlargement to maintain a constant shear rate at the interface between the flowing blood and endothelium,[7] with minimal hemodynamic compromise. When hemodynamically significant stenosis finally occurs, it probably represents an even later stage of atherogenesis, determined by pathways related to plaque rupture, intraplaque hemorrhage, thrombosis, and scarring.[1] Finally, clinical events such as stroke or myocardial infarction probably reflect determinants of arterial occlusion at the latest stages of atherogenesis, as well as determinants related to thrombosis and thrombolysis.

We have been acquiring longitudinal experience using 2-dimensional ultrasound to evaluate the total cross-sectional area of all carotid arterial plaques in an individual. This allowed us to define a quantitative trait termed *unexplained atherosclerosis*, which uses multivariate regression analysis with traditional risk factors as the independent variables to identify individuals whose total carotid plaque area was significantly greater than would be expected.[8–11] We also examined associations between total carotid plaque area and nontraditional atherosclerosis risk factors.[9–11] However, the relationship between traditional risk factors, nontraditional risk factors, and the more advanced trait of carotid stenosis has not been systemically studied. Furthermore, the associa-

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From Robarts Research Institute (J.D.S., R.A.H.), London, Ontario, Canada.

Correspondence to Dr J. David Spence, Robarts Research Institute, 1400 Western Rd, London, Ontario, Canada N6G 2V2. (E-mail dspence@robarts.ca) or Dr Robert Hegele, Blackburn Cardiovascular Genetics Laboratory, 406-100 Perth Drive, London, Ontario, Canada N6A 5K8 (E-mail hegele@robarts.ca).

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tion of risk factors with total carotid plaque area and carotid stenosis measured in the same individuals has not yet been assessed. In this study we compare the association between atherosclerosis risk factors and 2 different ultrasound-derived quantitative traits, namely, total carotid plaque area and carotid stenosis, measured concurrently in the same group of individuals.

Subjects and Methods

Study Sample

The patient sample included all patients from an inception cohort initiated in 1994, for whom complete data were available for the base multiple regression model. These patients were consecutive patients with baseline measurement of plaque from the Atherosclerosis Prevention Clinic and the Stroke Prevention Clinic of the London Health Sciences Centre, London, Canada. Each subject had baseline measurement of total carotid plaque area and carotid stenosis, as described below.

Risk Factors

Age and sex were self-reported by the patients and were supported by hospital records. We were not aware of any transgender patients in the cohort, but 1 patient had Klinefelter syndrome. We defined pack-years of smoking as number of packs per day of cigarettes smoked multiplied by the number of years smoked. Diabetes was defined as a medical diagnosis of diabetes and/or use of oral hypoglycemic agents and/or insulin. Use of medications for hypertension and dyslipidemia was similarly recorded.

Biochemical and Genetic Determinations

After a 12-hour fast, blood was taken for biochemical determinations. Plasma triglycerides and total and HDL cholesterol were determined as described.[8–12] Plasma total homocysteine was measured by high-performance liquid chromatography.[8,10] Lipoprotein(a) [Lp(a)] was measured with the use of an enzyme-linked immunosorbent assay [Macra Lp(a), Wampole Laboratories]. Genotypes of common polymorphisms in genes encoding methylenetetrahydrofolate reductase (*MTHFR* nucleotide 667, C677T), lipoprotein lipase (*LPL* codon 9, D9N), and paraoxonase-1 (*PON1* codon 192, Q192R) were determined as described[9,11,13] with the use of sequence-proven positive control samples on each gel and blinded repeats of 5% of samples, with no discrepancies noted.

Noninvasive Measurements

Total carotid plaque area was measured as described previously described[12,14] with the use of a high-resolution duplex ultrasound scanner (initially an ATL Mark 9; since 2000 an ATL 5000 HDI; Advanced Technology Laboratories). The same certified vascular ultrasound technologist made all measurements. Plaque was defined as a local thickening of the intima >1 mm. Measurements were made in magnified longitudinal views of each plaque seen in the right and left common, internal, and external carotid arteries. The plane for measurement of each plaque was chosen by scanning to find the largest extent of plaque. The image was then frozen and magnified, and the plaque was measured by tracing around the perimeter with a cursor on the screen. The microprocessor in the scanner then displayed the cross-sectional area of the plaque. The operator then moved on to the next plaque and repeated the process until all plaques were measured. Total carotid plaque area was defined as the sum of cross-sectional area of all plaques between the clavicle and angle of the jaw. Blinded intraobserver and interobserver intraclass correlations were 0.94 (n=50) and 0.85 (n=50), respectively.

Carotid stenosis was measured by Doppler peak frequency shift. Carotid stenosis was calculated as the sum of percent stenosis of left and right internal carotid arteries for each study subject. Carotid stenosis was calibrated against angiographic stenosis that had been measured in 212 carotid arteries in subjects from the North American Symptomatic Carotid Endarterectomy Trial.[13] The *R* for carotid stenosis determined angiographically and by ultrasound was 0.77.

TABLE 1. Baseline Characteristics of the Study Population

Continuous Variables	Mean±SD
Age, y	57.2±14.6
Systolic blood pressure, mm Hg	140±21
Diastolic blood pressure, mm Hg	80±12
Total cholesterol, mmol/L	5.12±1.09
Triglycerides, mmol/L	1.86±1.25
HDL cholesterol, mmol/L	1.28±0.43
LDL cholesterol, mmol/L	3.20±1.13
Lipoprotein(a), g/L	0.16±0.20
Total homocysteine μmol/L	12.5±7.1
Total carotid plaque area, cm^2	0.87±1.06
Carotid stenosis, %	46.1±30.0

Categorical Variables	Percent
Females	47.1%
Diabetes mellitus	8.9%
On lipid-lowering drugs	49.6%
On antihypertensive drugs	57.2%
Previous myocardial infarction	25.2%
Previous stroke	19.5%
Previous transient ischemic attack	27.6%
Former smokers (no smoking for 1 year)	32.4%
Current smokers	12.4%

Statistical Methods

Data were entered and analyzed with the use of SPSS PC +10.5 (SPSS). Multiple regression analysis was performed by a cube root transformation of total carotid plaque area, which gave a distribution that was not significantly different from normal, and on untransformed carotid stenosis, which had a distribution not significantly different from normal. The independent variables were ones that we previously showed to be significant independent predictors of baseline total carotid plaque area.[8–12] These included age, sex, pack-years of cigarette smoking (for current and former smokers), medical diagnosis of diabetes (almost always type 2 diabetes), systolic blood pressure at baseline, plasma total cholesterol concentration at baseline, pharmacological treatment of dyslipidemia, and pharmacological treatment of hypertension.[8–12]

These regression models were used as basal models. β-Coefficients and significance levels for specific variables were compared for total carotid plaque area and carotid stenosis. Next, for each model, multiple regression analysis was performed again, with forced entry of each new independent variable separately. Baseline plasma concentrations of Lp(a) and total homocysteine and genotypes for *MTHFR* C677T, *LPL* D9N, and *PON1* Q192R (assuming recessive effects for the 677T, N9, and R192 alleles) were forced into each basal regression model. A priori, we hypothesized that any potential effect of *LPL* and *PON1* genotypes would occur at earlier stages of atherogenesis, while any potential effect of Lp(a), total homocysteine, and *MTHFR* genotype would occur at later stages of atherogenesis.[1]

Results

Baseline Attributes of Patients

The baseline clinical and biochemical attributes of the 1821 study patients are shown in Table 1. Approximately half of

TABLE 2. Multiple Regression Model for Baseline Total Carotid Plaque Area

Independent Variable	Standardized β Coefficient	*P* Value
Age	0.533	<0.0001
Sex (M=0, F=1)	−0.131	<0.0001
Pack-years of smoking	0.184	<0.0001
Diabetes mellitus	0.066	<0.0001
Baseline systolic blood pressure	0.102	<0.0001
Baseline total cholesterol	0.053	0.002
On lipid therapy	0.110	<0.0001
On antihypertensive therapy	0.042	0.023

Model R^2=0.526; P<0.0001.

the subjects were female, and approximately two thirds of subjects had a previous cardiovascular or cerebrovascular event. The majority of subjects were receiving treatment for hypertension and/or dyslipidemia.

Determinants of Total Carotid Plaque Area and Carotid Stenosis

The Pearson correlation coefficient between total carotid plaque area and carotid stenosis in this data set was 0.598 (P<0.0001). Tables 2 and 3 show the regression coefficients for the independent variables used in the regression models for total carotid plaque area and carotid stenosis, respectively. The portfolio of independent variables modeled for each trait included age, sex, systolic blood pressure, serum total cholesterol, pack-years of smoking, treatment of dyslipidemia, and treatment of hypertension. Regression modeling gave R^2 values of 0.526 and 0.134 for total carotid plaque area and carotid stenosis, respectively (both P<0.0001). As a group, the risk variables that we previously found to be associated with total carotid plaque area in smaller study samples were also strongly associated with total carotid plaque area in the present study sample, as indicated by the moderately large R for the total model. However, this same group of risk variables was markedly less strongly associated with carotid stenosis, as indicated by the smaller R values for the total model.

Traditional risk factors had different relationships with total carotid plaque area and carotid stenosis, as seen in Tables 2 and 3. For instance, diabetes was significantly

TABLE 3. Multiple Regression Model for Baseline Carotid Stenosis

Independent Variable	Standardized β Coefficient	*P* Value
Age	0.256	<0.0001
Sex (M=0, F=1)	0.147	<0.0001
Pack-years of smoking	0.184	<0.0001
Diabetes mellitus	−0.021	NS (0.51)
Baseline systolic blood pressure	0.131	<0.0001
Baseline total cholesterol	−0.015	NS (0.64)
On lipid therapy	0.082	0.012
On antihypertensive therapy	0.042	NS (0.24)

Model R^2=0.134; P<0.0001.

TABLE 4. Multiple Regression Model for Baseline Total Carotid Plaque Area, Adding Individual Nontraditional Risk Factors to Model in Table 2

Independent Variable Added	Standardized β Coefficient	*P* Value
Plasma homocysteine	0.095	<0.0001
Plasma lipoprotein(a)	0.003	NS (0.92)
LPL D9N genotype	0.150	0.003
MTHFR C677T genotype	0.001	NS (0.99)
PON1 Q192R genotype	−0.002	NS (0.98)

associated with total carotid plaque area but not with carotid stenosis. Additionally, both baseline total cholesterol concentration and treatment for dyslipidemia were associated with total carotid plaque area, but only treatment for dyslipidemia was significantly associated with carotid stenosis. Similarly, baseline systolic blood pressure and pharmacological treatment of hypertension were each significantly associated with total carotid plaque area, but only baseline systolic blood pressure was significantly associated with carotid stenosis. Furthermore, the magnitude of the association for individual variables as estimated by the β-coefficients of regression was different between the 2 phenotypes. For instance, age accounted for approximately 53% of the explained variance of total carotid plaque area but only approximately 26% of the explained variance in carotid stenosis. In addition, female sex was inversely associated with total carotid plaque area, accounting for approximately 13% of the explained variance, but was positively associated with carotid stenosis, accounting for approximately 15% of the explained variance.

Tables 4 and 5 show results of regression modeling for nontraditional risk factors that were added individually to the basal models for each phenotype. Plasma homocysteine concentration was significantly associated with total carotid plaque area but not carotid stenosis. In contrast, plasma Lp(a) concentration tended to be associated with carotid stenosis but not with total carotid plaque area. Genotype of *LPL* D9N was significantly associated with total carotid plaque area, as reported,[11] but also with carotid stenosis. Specifically, homozygosity for N9/N9 accounted for an additional 15% and 17% of the explained variance of total carotid plaque area and carotid stenosis, respectively. Genotype of *MTHFR* C677T was not associated either with total carotid plaque area, as previously reported,[10] or with carotid stenosis. Finally, genotype of *PON1* Q192R tended to be associated with carotid

TABLE 5. Multiple Regression Model for Baseline Carotid Stenosis, Adding Individual Nontraditional Risk Factors to Model in Table 3

Independent Variable Added	Standardized β Coefficient	*P* Value
Plasma homocysteine	0.035	NS (0.18)
Plasma lipoprotein(a)	0.076	NS (0.08)
LPL D9N genotype	0.172	0.007
MTHFR C677T genotype	0.018	NS (0.76)
PON1 Q192R genotype	0.119	NS (0.06)

stenosis but not with total carotid plaque area. Specifically, homozygosity for R192/R192 accounted for an additional 12% of the explained variance of carotid stenosis.

Discussion

In 1821 subjects, we studied 2 related atherosclerosis phenotypes in the same vascular bed, namely, carotid arterial plaque area and carotid stenosis, each measured using ultrasound imaging. We found that (1) these 2 traits were only moderately well correlated ($r \approx 0.6$); (2) traditional atherosclerosis risk factors explained a greater proportion of the variance in total carotid plaque area than in carotid stenosis ($\approx$53% versus $\approx$13%); and (3) associations of both traditional and nontraditional risk factors differed substantially between total carotid plaque area and carotid stenosis. The findings suggest that ultrasound-derived total carotid plaque area and carotid stenosis measured in the same individuals, while related, represent distinct intermediate traits with unique determinants and relationships to atherosclerosis risk factors. Thus, the use of one trait or the other as a surrogate for "atherosclerosis" would lead to different conclusions regarding the role of specific risk factors in a particular patient sample.

While there are important limitations to cross-sectional studies, including inability to reflect temporal changes of risk factors, noninvasive phenotypes of atherosclerosis appear to predict long-term outcome, albeit inconsistently. Because the Framingham factors account only incompletely for cardiovascular risk,[15] noninvasive measures have been considered as having the potential to provide a useful increment in risk assessment because they may be more direct markers of the disease process. While IMT is the best-studied noninvasive phenotype, its relationship to disease end points ranges from relatively modest[16,17] to moderate.[18,19] Quantification of carotid plaques is a relatively new approach, with even less evaluation with respect to predicting end points. We found that total carotid plaque area was a strong predictor of stroke, death, and myocardial infarction.[14] After adjustment for risk factors, including total homocysteine, patients in the top quartile of plaque area had a relative risk of 3.5 compared with the lowest quartile.[14] We also showed, after adjusting for the same panel of risk factors, that patients with progression of plaque had twice the risk of those with stable plaque or regression.[14] We have also found that stenosis was not a strong predictor of those outcomes.[20] In contrast, IMT was a stronger predictor of stroke than of myocardial infarction,[19] while total plaque area was a stronger predictor of myocardial infarction than of stroke.[14] The data, while indirect, suggest that IMT, total carotid plaque area, and carotid stenosis likely assay different aspects of atherosclerosis.

While the simple correlation between total carotid plaque area and carotid stenosis was statistically highly significant, the $r \approx 0.6$ indicated that this correlation was only moderate. In retrospect, this is not that surprising because total carotid plaque area is related to the carotid arterial wall, while carotid stenosis is related to factors leading to plaque rupture. We speculate that such factors include a thin cap, inflammation, and other factors predisposing to rupture, as well as hemodynamic factors in the lumen, such as high or low shear and shear oscillations. High velocities over the surface of the plaque, by the Bernoulli principle, effectively create suction on the cap of the plaque. Constantinides[21] has discussed the interaction between plaque rupture and thrombosis. It is possible to imagine situations in which total carotid plaque area is large, but the plaques themselves are thin and diffuse, with little resultant hemodynamic compromise. Conversely, some small isolated focal plaques might protrude markedly into the arterial lumen, producing a hemodynamic effect that affects carotid stenosis determination. Furthermore, according to the concept of compensatory enlargement, plaque in its early development does not cause stenosis because the artery enlarges in order to maintain a constant luminal diameter.[7] The purpose of such compensatory enlargement may be to maintain a constant shear rate at the interface between flowing blood and the endothelium.[7] Nitric oxide production may account for plaque growth away from high shear zones, while endothelin production may account for plaque tending to fill in low shear zones.[22] The relationship between plaque growth and oscillatory shear, as well as other hemodynamic patterns, is actively being studied with MRI and computational flow modeling.[23] By comparison, carotid stenosis develops later in atherogenesis as a consequence of intraplaque events such as hemorrhage or rupture, with subsequent scarring and interference with flow. Thus, not all plaques produce stenosis.

However, total carotid plaque area and carotid stenosis are both surrogate biomarkers for the atherosclerotic process. Thus, it is not surprising that they share common determinants, such as age, sex, smoking, baseline systolic blood pressure, and treatment for dyslipidemia. However, the differences between the risk factor determinants of total carotid plaque area and carotid stenosis seen in regression analysis may be informative and hypothesis generating. For instance, total carotid plaque area is essentially a quantitative arterial wall trait and thus may be more closely associated with determinants of earlier stages of atherosclerosis, such as endothelial dysfunction, lipid infiltration, and foam cell formation.[1] In contrast, carotid stenosis is essentially a quantitative arterial lumen trait that appears to be less strongly associated with those factors since they accounted for much less of its variation in the regression model. Stronger association of carotid stenosis with factors related to hemodynamics, scarring, and thrombosis may explain this, but reliable assays for such factors have yet to be proven. The opposite association between sex and the 2 imaging traits is also of interest, suggesting that male sex is more closely associated with plaque growth but that female sex is more closely associated with a difference in arterial size, unfavorable arterial hemodynamics, or differences in remodeling. This issue has been explored in another study; at any age, men had more plaque and less stenosis than women.[20] In addition, the absence of association between carotid stenosis and diabetes indicates that the influence of diabetes on carotid stenosis may not be independent of other variables. The association between carotid stenosis and systolic blood pressure at baseline but not with treatment for hypertension is consistent with a more direct relationship with a quantitative hemodynamic arterial trait. The association between carotid

stenosis and treatment for dyslipidemia but not with cholesterol concentration at baseline is consistent with a more chronic relationship with hyperlipidemia that is independent of a specific quantitative determination of cholesterol.

With respect to the nontraditional risk factors, the specific significant association between plasma total homocysteine concentration and total carotid plaque area but not carotid stenosis would seem to be consistent with a predominant role for this metabolite in plaque growth but not with plaque rupture or scarring. The trend toward a significant association between plasma Lp(a) concentration and carotid stenosis, but not total carotid plaque area, may be consistent with a predominant role for this lipoprotein through an effect on thrombosis or scarring of ruptured plaques. With respect to *LPL* codon 9 genotype, we previously showed this to be associated with total carotid plaque area in another study sample.[11] The association with both total carotid plaque area and carotid stenosis suggests that whatever the functional consequence of the codon 9 variation in *LPL*, it is associated with the 2 distinct atherosclerosis phenotypes measured in this study. The absence of association between *MTHFR* genotype and both total carotid plaque area and carotid stenosis is consistent with our previous observation that plasma homocysteine concentration, but not *MTHFR* genotype, is associated with vascular phenotypes.[9] This may be explained by the many other causes of elevated total homocysteine besides the *MTHFR* genotype.

The results indicate that within the same study sample, atherosclerosis determinants differ in their relationships with particular phenotypes, even when these are correlated with each other and determined with the use of similar imaging modalities in the same vascular bed. The variability of the associations between total carotid plaque area and carotid stenosis was seen for both traditional and nontraditional atherosclerosis risk factors. This has implications for studies of determinants of "atherosclerosis" that use indirect surrogate markers determined noninvasively. Different phenotypes of atherosclerosis should not be regarded as equivalent. While arterial wall plaque burden as measured by total carotid plaque area bears a general relationship with change in luminal hemodynamics as measured by carotid stenosis, there are substantial differences in the pathogenesis of each trait. They probably reflect influences of different risk factors and different gene products, and distinctions among them will be important in understanding atherogenesis. The major contribution of age and sex to variance in total carotid plaque area and carotid stenosis means that these phenotypes may be relatively insensitive to new factors contributing to atherosclerosis. For this reason we are now exploring phenotypes on the basis of the rate of progression of total carotid plaque area and carotid stenosis. In these models, age is less significant a predictor of rate of progression, suggesting that these traits may be more sensitive to detect new determinants of atherosclerosis.

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