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Plasma and serum L-selectin and clinical and subclinical the Multi-Ethnic Study of Atherosclerosis (MESA)cardiovascular disease

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Abstract

L-selectin has been suggested to play a role in atherosclerosis. Previous studies on cardiovascular disease (CVD) and serum or plasma L-selectin are inconsistent. The association of serum L-selectin (sL-selectin) with carotid intima-media thickness, coronary artery calcium, ankle-brachial index (subclinical CVD) and incident CVD was assessed within 2403 participants in the Multi-Ethnic Study of Atherosclerosis (MESA). Regression analysis and the Tobit model were used to study subclinical disease; Cox Proportional Hazards regression for incident CVD. Mean age was 63 ± 10 , 47% were males; mean sL-selectin was significantly different across ethnicities. Within each race/ethnicity, sL-selectin was associated with age and sex; among Caucasians and African Americans, it was associated with smoking status and current alcohol use. sL-selectin levels did not predict subclinical or clinical CVD after correction for multiple comparisons. Conditional logistic regression models were used to study plasma L-selectin and CVD within 154 incident CVD cases, occurred in a median follow up of 8.5 years, and 306 age-, sex-, and ethnicity-matched controls. L-selectin levels in plasma were significantly lower than in serum and the overall concordance was low. Plasma levels were not associated with CVD. In conclusion, this large multi-ethnic population, soluble L-selectin levels did not predict clinical or subclinical CVD.

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Keywords

atherosclerosis; cardiovascular diseases; ethnic groups; L-selectin

L-selectin is an adhesion protein constitutively expressed on neutrophils and lymphocytes and is involved in the early phase of leukocyte-endothelial interaction during inflammation. Circulating levels are the result of proteolytic cleavage upon activation.¹ The inhibition of this cleaving process increases neutrophil and leukocyte adhesion to inflammatory sites.² In addition, circulating L-selectin was found to maintain functional activity preventing leukocytes from interacting with L-selectin ligands on endothelial cells.³ Taken together, these results support the hypothesis that circulating L-selectin has a protective role in the inflammatory process.

As atherosclerosis is primarily an inflammatory disease, the involvement of L-selectin in its pathogenesis is biologically plausible. While a protective role of the cell-bound portion of this protein has been demonstrated in animal models,⁴ human studies investigating circulating L-selectin and cardiovascular disease (CVD) are inconsistent. The latter appear to be complicated by whether the protein was measured in serum or plasma samples.

Lower levels of serum L-selectin were observed in patients with cardiovascular risk factors compared to controls.⁵ Likewise, serum levels of this protein were inversely associated with carotid plaque size among elderly patients.⁶ Finally, serum L-selectin was found to be lower in coronary heart disease (CHD)^{7,8} and peripheral artery disease (PAD)⁹ patients compared to controls. Collectively these studies are in keeping with previous *in vitro* and *in vivo* evidence of a protective role of this circulating protein in atherosclerotic disease.

Conversely, other studies conducted in serum samples showed that higher levels of L-selectin were associated with acute stroke,¹⁰ and no difference in serum L-selectin levels was observed between PAD and CHD cases compared to controls.^{11,12} In addition, in plasma, L-selectin levels were higher in patients with unstable angina^{13–15} and PAD¹⁶ compared to controls and levels were lowered by statin therapy in a group of hyperlipidemic patients,¹⁷ suggesting a pro-inflammatory role of the circulating protein.

Such inconsistencies might be due to methodological complexities (eg, serum or plasma, case-control design), as well as to differences in population characteristics and highlight the challenges of using soluble protein measurements as a surrogate in understanding the relationship of membrane-bound proteins and atherosclerosis.

Furthermore, the subjects studied previously were highly selected from clinical populations and predominantly white, underscoring the need of a broader and multiethnic study cohort in order to obtain reliable and generalizable observations. The role of soluble L-selectin in atherosclerotic disease and the relationship of protein levels with cardiovascular risk factors need to be clarified using a rigorous, population-based approach.

Therefore, the goal of this study was to determine the role of circulating L-selectin as a biomarker of subclinical CVD and as a predictor of CVD events in a large, diverse

population, investigating both serum and plasma L-selectin levels. In order to complete this goal, we first conducted a population-based study to investigate the association of serum levels of L-selectin and clinical and subclinical CVD among four race/ethnicities. Second, a nested case-control study was performed to compare serum and plasma levels of L-selectin and association with CVD in participants who experienced a cardiovascular event compared to age (± 1 year), sex, and race/ethnicity matched controls.

MATERIALS AND METHODS

The Multi-Ethnic Study of Atherosclerosis (MESA) enrolled 6,814 participants from 2000–2002 in six field centers located in Baltimore, MD, Chicago, IL, Forsyth County, NC, Los Angeles County, CA, northern Manhattan, NY, and Saint Paul, MN. This population had no history of clinical CVD, was aged 45–84 and included 38% Non-Hispanic white, 28% African, 22% Hispanic, and 12% Chinese Americans. The MESA study has been described in detail elsewhere.¹⁸ Under the auspices of the Multi-Scale Biology of Atherosclerosis in the Cellular Adhesion Pathway (HL98077 – MESA Adhesion Study), a stratified random sample of 2403 MESA participants (~600 of each race/ethnicity) free of CVD at exam 2 who had serum and plasma available were used in the analyses.

Information on demographics, cardiovascular risk factors, past medical history and co-morbidities, social history, family history, and medications were collected through a combination of self-administered questionnaires and interview-administered questionnaires for all participants. BMI was calculated as weight (kg)/height² (m²). Resting seated blood pressure was measured three times and the average of the second and third readings was used in the analyses. Diabetes was defined as any participant who self-reported a physician diagnosis, used diabetic medication, had a fasting glucose ≥ 126 mg/dL, or a non-fasting glucose of ≥ 200 mg/dL. Hypertension was defined according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) guidelines¹⁹ as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or use of anti-hypertensive medications. Triglycerides were measured in plasma by a glycerol blanked enzymatic method, and cholesterol was measured in plasma using a cholesterol oxidase method. HDL cholesterol was measured by the cholesterol oxidase method after precipitation of non-HDL-cholesterol with magnesium/dextran. LDL-cholesterol was calculated in specimens having a triglyceride < 400 mg/dL via the Friedewald equation.

Computed tomography to obtain coronary artery calcium (CAC) and common carotid ultrasound to measure intima-media thickness (IMT) were performed as previously described.^{20,21} Ankle brachial index (ABI) was defined as the ratio of ankle to arm blood pressure; systolic blood pressure measurements were obtained from bilateral brachial, dorsalis pedis and posterior tibial arteries.²²

Circulating L-selectin was measured in both frozen serum and plasma by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using the Human soluble L-selectin/CD62L Immunoassay kit (R&D Systems, Minneapolis, MN). The inter-assay coefficient of variation of the MESA laboratory when serum was measured was 6.7% at a

mean concentration of 943 ng/mL for an in-house serum pooled control 0, and 7.9% at a mean concentration of 866 ng/mL for a lyophilized control, respectively. The inter-assay coefficient of variation of the MESA laboratory when plasma was measured was 3.8% at a mean concentration of 1196 ng/mL for an in-house serum pooled control P, and 5.5% at a mean concentration of 1002 ng/mL for a lyophilized control, respectively. The manufacturer's minimum detection level is 0.3 ng/mL. The collection and storage of serum and plasma samples was performed according to a standardized protocol across the participating centers.¹⁸

Public files (death certificates), medical records from hospitalizations, autopsy reports, and interviews from participants, and in some instances, interviews or questionnaires from physicians, relatives, or friends were used to ascertain cardiovascular events defined as myocardial infarction (MI), resuscitated cardiac arrest, definite angina, probable angina-if followed by revascularization, stroke, stroke death, coronary heart disease (CHD) death, other atherosclerotic death, and other CVD death. Detailed events ascertainment methods have been previously described.²³

Participant characteristics were compared across race/ethnicities using analysis of variance (Kruskal-Wallis) for continuous variables and the chi-square test (exact) for categorical variables. Regression analysis was used to assess the association of L-selectin levels with IMT or ABI. Participants with ABI >1.3 were excluded from the analysis.

Because the distribution of CAC has a large percentage of zero measurements, the Tobit model was used to investigate the relationship between soluble L-selectin and CAC. The Tobit model is a form of linear regression that treats the outcome measure as a latent variable comprised of both a discrete and continuous component. The outcome measure equals the latent variable whenever the latent variable is above zero, and zero otherwise. Cox proportional hazard regression models were used to assess the association between serum L-selectin and CVD. Follow-up time was calculated from the visit 2 date to the time of CVD or date of last follow-up. Subjects were censored on the date last known to be event free. The protein association with clinical and subclinical CVD was assessed both for the four races/ethnicities pooled together and separately via stratified analysis; in the fully adjusted model age, sex, BMI, smoking status, alcohol use, LDL, HDL, triglycerides, hypertension, and diabetes were included. To account for multiple comparisons, we used a Bonferroni correction and set a threshold of $P < 0.01$ ($0.05/4$) to determine statistical significance based on the total number of outcomes assessed (3 subclinical measures and CVD events). For the case-control study, plasma and serum L-selectin levels were compared between cases and controls using conditional logistic regression models, taking into account the matched set study design. The concordance between L-selectin plasma and serum levels was assessed using Bland-Altman plots.

This study was conducted according to the principles of the Declaration of Helsinki, informed consent was obtained from all participants. The Mayo Clinic Institutional Review Board approved the study.

RESULTS

We included a total of 2403 subjects (579 African, 600 Chinese, 620 non-Hispanic white and 604 Hispanic Americans) with available serum and plasma samples. In our population, mean age was 63 ± 10 and 47% were males; no significant differences in age or sex were observed across ethnicities. Significant race/ethnic differences in BMI, total and HDL cholesterol, triglycerides, diabetes, and hypertension but not LDL cholesterol were observed (Supplemental Table I). Similarly, subclinical disease prevalence was different across races/ethnicities as previously described within the MESA population. During a median follow up of 8.5 years, 154 individuals (36 African, 21 Chinese, 50 non-Hispanic white and 47 Hispanic Americans) experienced a CVD event.

Serum L-selectin levels were significantly associated with race/ethnicity, with non-Hispanic whites having the highest mean level (mean $\pm$ SD 956 ± 215 ng/mL) followed by Hispanics (mean $\pm$ SD 904 ± 195 ng/mL), African (mean $\pm$ SD 867 ± 192 ng/mL), and Chinese (mean $\pm$ SD 834 ± 174 ng/mL) Americans ($P < 0.001$). Serum L-selectin was inversely correlated with age and levels were lower in males compared to females in every race/ethnicity ($P < 0.002$ for each group) as shown in Table I. In addition, L-selectin levels were significantly lower in current smokers and in current alcohol users among non-Hispanic whites and African Americans.

Serum L-selectin was not associated with any of the subclinical disease measurements (i.e., CAC, ABI, or IMT) in any race/ethnicity. Similarly, in the adjusted model, serum L-selectin was not significantly associated with overall CVD in any of the four race/ethnicities (Table II). In non-Hispanic whites, serum L-selectin was marginally related to CVD events with higher levels associated with increased risk of CVD ($P = 0.04$), but when adjusted for multiple comparisons, this result was non-significant.

Among the subgroup of 154 cases and 306 controls, we observed significantly lower mean plasma L-selectin levels compared to serum levels (791.0 ± 176.5 ng/mL vs 877.9 ± 212.6 ng/mL, respectively) and plasma levels were once again significantly different across races/ethnicities ($P < 0.001$). In addition, the overall concordance of serum and plasma levels using Bland-Altman plot was low in our population, indicating that the difference between serum and plasma levels varied widely among individuals (Fig 1). Nonetheless, this variation was not significantly different by ethnicity or by case-control status. Furthermore, neither serum nor plasma L-selectin levels were significantly associated with incident CVD (serum OR, 1.01; 95% CI, 0.82–1.26; $P = 0.90$; plasma OR, 0.99; 95% CI, 0.79–1.23; $P = 0.89$).

DISCUSSION

Previous studies investigating the role of circulating L-selectin as a biomarker of subclinical CVD and as a predictor of CVD events have reported conflicting results. In order to understand the contribution of study design, sample type, and population characteristics to the inconsistent results, we assessed the association of L-selectin measured in serum and plasma with subclinical and clinical CVD in a large, multiethnic population, free of CVD at

baseline. Using the large and diverse study population within MESA, we observed that serum and plasma L-selectin levels were significantly different among race/ethnicities and plasma levels were lower than serum levels. However, circulating L-selectin, whether measured in plasma or serum, was not associated with subclinical or clinical CVD in this large and diverse population.

L-selectin is constitutively expressed on leukocytes surface, and its role in mediating cell adhesion during the atherosclerotic process is controversial. Previous studies *in vivo* suggested a facilitating role of the membrane-bound form of this adhesion protein in leukocyte accumulation during atherogenesis;^{24,25} on the contrary, in animal models of atherosclerosis lacking L-selectin, a protective role of the protein during early phases of plaque formation was observed.⁴ The soluble portion of L-selectin, formed by protease shedding, is easily measurable in serum or plasma, but its specific function in atherosclerosis is difficult to interpret. In fact, circulating L-selectin was found to be protective in some studies,⁷⁻⁹ and a risk factor in others.^{10,13-16} The previous body of literature is almost exclusively represented by case-control studies from predominantly Caucasian clinical populations with only a single L-selectin measurement (ie, serum or plasma).

In contrast to a subset of prior results, our study did not observe any significant associations between soluble L-selectin, measured in either serum or plasma, and clinical or subclinical CVD in any of the four races/ethnicities. This could be due to a variety of reasons. First, given the paucity of previous data on levels of soluble L-selectin, unknown confounders might have played a role in determining present results. Secondly, the expression of the protein on the leukocyte surface wasn't directly assessed in this study and the circulating form might have no function after cleavage. Finally, these results suggest the lack of function of soluble L-selectin in the pathogenesis of atherosclerosis.

Due to the longitudinal design and to the population diversity, this study was able to assess the association of L-selectin levels with a variety of demographic and risk factors. We described significant differences in mean L-selectin levels across races/ethnicities. This is a novel finding and might be due to genetic determinants, as was previously demonstrated for intracellular adhesion molecule-1 (ICAM-1)²⁶ or to social or cultural factors. We confirmed the inverse association between age and L-selectin levels that was previously reported in a predominately Caucasian population by Ponthieux et al. in the Stanislas cohort, a family study conducted in France.²⁷ In contrast with the findings of this study, levels of soluble L-selectin were higher in women compared to men across races/ethnicities in our population. In addition, we reported lower levels of this protein among current alcohol users and current smokers in non-Hispanic white and African Americans, but not among Chinese and Hispanics.

In this study, significantly lower levels of L-selectin in plasma were observed compared to levels in serum and the overall concordance of serum and plasma levels was low, in contrast with that reported in small and very selected populations.^{3,28} As this protein was rarely measured in large populations, and had never been measured in both serum and plasma, reasons for this discrepancy are unknown. Nevertheless, at least with regard to CVD, the observed differences in the protein levels seem to have no impact on the association with the

outcome. The comparison across previous studies results utilizing different samples (serum vs plasma) might present some challenges due to the inconsistencies in protein levels.

Our study has several strengths including a large population-based cohort from four race/ethnic groups. In addition, by measuring both serum and plasma L-selectin in a single study cohort, we were able to provide evidence that previous inconsistencies in the association of the protein with CVD were not due to the sample type as neither measure was associated with atherosclerosis in our population. Finally, the inclusion of subclinical endpoints allowed us to investigate the role of L-selectin in early atherosclerosis, as suggested by previous animal models.⁴ We acknowledge limitations of this study. The number of clinical events in each ethnic group might not be sufficient to observe ethnic-specific associations of CVD events with soluble L-selectin. Nevertheless, results for events in pooled analyses and for subclinical outcomes, which have been demonstrated to be predictive of clinical CVD and were available in the whole population, support the lack of association observed. Furthermore, as mentioned above, our study did not measure functional cellular-bound L-selectin; thus we are unable to make any inferences in regards to the relationship of cellular bound L-selectin and atherosclerosis. In particular, in animal models, a slow turnover of soluble L-selectin have been documented, suggesting that elevated circulating protein levels might reflect a long half-life of the protein, more than an intense leukocyte activation.²⁹

This is the first large, multi-ethnic study investigating the role of circulating L-selectin in predicting clinical and subclinical CVD. We believe that this study contributes significantly to the body of knowledge on soluble L-selectin levels, their determinants and possible challenges in their measurements. Despite the biological plausibility of L-selectin involvement in the pathogenesis of atherosclerosis, the circulating form of this protein was not found to be associated with clinical or subclinical CVD in any of the four race/ethnic groups studied.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ABI	ankle-brachial index
CAC	coronary artery calcium

CHD	coronary heart disease
CVD	cardiovascular disease
ELISA	enzyme-linked immunosorbent assay
ICAM-1	intracellular adhesion molecule-1
IMT	intima –media thickness
MESA	Multi-Ethnic Study of Atherosclerosis
MI	myocardial infarction
PAD	peripheral artery disease

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Brief Commentary

Background

L-selectin mediates the adhesion of leukocytes to endothelial cells during inflammation and it has been hypothesized to play a role in atherosclerosis. The association of protein levels with cardiovascular outcomes is controversial. In addition, previous studies on this protein were conducted on predominantly white populations.

Translational significance

Given the striking ethnic differences in the prevalence of cardiovascular disease, a better understanding of the underlying mechanisms of atherosclerosis is needed to promote effective prevention strategies. The goal of this study is to assess the value of L-selectin in predicting subclinical and clinical cardiovascular disease in a large and diverse population.

Fig. 1.
Serum and plasma L-selectin levels concordance in the case-control population.

Mean levels of soluble L-selectin (ng/mL) by ethnicity for traditional cardiovascular risk factors measured at MESA exam 2

Table 1

	Non-Hispanic white American	Chinese American	African American	Hispanic American	Interaction <i>P</i> value
Sex					0.06
Male	n=298 913.01 (200.97)	n=296 802.67 (171.10)	n=262 822.02 (188.72)	n=276 941.01 (178.40)	
Female	n=322 995.09 (219.69)	n=304 864.87 (172.57)	n=317 904.67 (187.12)	n=328 956.43 (193.81)	
<i>P</i> value	<.0001	<.0001	<.0001	<.0001	
Age categories, years					0.74
45–49	n=61; 1004.63 (199.58)	n=53 863.83 (135.52)	n=44; 960.65 (204.92);	n=64 922.35 (179.29)	
50–54	n=99 1006.86 (211.43)	n=91 879.40 (160.50)	n=104 896.38 (182.93)	n=89 920.62 (205.88)	
55–59	n=104 951.48 (187.05)	n=93 842.41 (154.08)	n=94 868.68 (201.82)	n=107 939.19 (202.25)	
60–64	n=71 986.19 (225.04)	n=86 844.05 (142.86)	n=86 904.21 (188.36)	n=99 918.43 (186.94)	
65–69	n=95 928.19 (199.84)	n=98 793.03 (159.07)	n=95 827.79 (178.80)	n=90 868.50 (193.07)	
70–74	n=91 910.89 (201.61)	n=83 806.58 (185.09)	n=74 847.49 (177.49)	n=62 920.57 (180.85)	
75	n=98 923.97 (256.32)	n=96 824.02 (236.15)	n=82 813.81 (196.07)	n=93 840.89 (195.13)	
<i>P</i> value	0.0006	0.0025	<.0001	<.0001	
Smoking status					0.12
Never	n=256 980.08 (204.54)	n=452 850.28 (182.28)	n=245 904.94 (191.19)	n=317 919.79 (187.11)	
Former	n=272 955.22 (223.72)	n=113 792.48 (139.26)	n=229 841.54 (181.03)	n=218 884.59 (192.67)	
Current	n=90 891.83 (201.93)	n=34 765.70 (129.67)	n=99 839.55 (207.17)	n=65 896.18 (235.34)	
<i>P</i> value	0.0006	0.0831	0.0121	0.9501	
Alcohol use					0.04
No	n=184 995.14 (231.51)	n=417 837.08 (165.97)	n=309 895.84 (187.66)	n=343 923.46 (189.15)	

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	Non-Hispanic white American	Chinese American	African American	Hispanic American	Interaction <i>P</i> value
Yes	n=435 938.96 (205.42)	n=182 828.85 (192.64)	n=267 836.37 (192.47)	n=260 879.01 (199.36)	
<i>P</i> value	0.0039	0.8316	0.0013	0.2106	

Abbreviation: SD, standard deviation.

Values are n; mean (SD). *P* values test association of risk factor with L-selectin adjusting for age and sex when appropriate. Interaction *P* value tests the risk factor by ethnicity/race interaction.

Table II

Association of serum L-selectin levels (ng/mL) and subclinical and clinical disease (Beta Per SD of L-Selectin Level for the Population = 200 ng/mL)

Outcome	Non-Hispanic white American			Chinese American			African American			Hispanic American			Overall		
	Beta (SE)	P		Beta (SE)	P		Beta (SE)	P		Beta (SE)	P		Beta (SE)	P	
CAC*															
Model 1	22.3 (31.1)	0.47		-61.0 (28.1)	0.03		2.1 (39.2)	0.96		-15.4 (30.8)	0.62		-14.1 (16.2)	0.39	
Model 2	39.1 (32.3)	0.23		-55.5 (27.8)	0.05		36.0 (41.1)	0.38		-16.6 (30.4)	0.58		-1.0 (16.5)	0.95	
ABI[†]															
Model 1	0.007 (0.004)	0.11		0.003 (0.004)	0.52		0.007 (0.006)	0.24		0.005 (0.005)	0.28		0.006 (0.002)	0.01	
Model 2	0.003 (0.004)	0.50		0.001 (0.004)	0.88		0.008 (0.006)	0.16		0.005 (0.005)	0.32		0.005 (0.002)	0.06	
IMT[‡]															
Model 1	-0.006 (0.007)	0.39		-0.004 (-0.009)	0.61		-0.012 (0.009)	0.20		0.012 (0.008)	0.14		-0.003 (0.004)	0.51	
Model 2	-0.006 (0.008)	0.42		0.0004 (0.009)	0.96		-0.012 (0.010)	0.22		0.008 (0.009)	0.32		-0.003 (0.004)	0.50	
CVD	HR (CI)	P		HR (CI)	P		HR (CI)	P		HR (CI)	P		HR (CI)	P	
Model 1	1.28 (1.01, 1.63)	0.04		0.89 (0.56, 1.43)	0.63		0.77 (0.52, 1.14)	0.19		0.99 (0.72, 1.37)	0.96		1.03 (0.87, 1.21)	0.75	
Model 2	1.27 (1.01, 1.60)	0.04		0.94 (0.58, 1.52)	0.80		0.75 (0.49, 1.13)	0.17		0.97 (0.69, 1.38)	0.89		1.03 (0.88, 1.22)	0.71	

Abbreviations: ABI, ankle-brachial index; CAC, coronary artery calcium; CI, confidence interval; CVD, cardiovascular disease; HR, hazards ratio; IMT = intima-media thickness; SE, standard error

Model 1: age/sex adjusted; Model 2: age, sex, BMI, smoking status, alcohol use, LDL, HDL, triglycerides, hypertension and diabetes. Ethnicity/race is included as a covariate in the overall models. The ethnicity/race by L-selectin interaction p-value for CAC was 0.58 (model 1) and 0.42 (model 2); for ABI 0.61 (model 1) and 0.40 (model 2); for IMT 0.10 (model 1) and 0.20 (model 2); for CVD 0.066 (model 1) and 0.069 (model 2).

* Mean CAC Agatston score $\pm$ SD was 211.1 ± 530.7 , 126.8 ± 356.6 , 147.9 ± 480.2 and 153.7 ± 417.3 ($P < 0.001$) for Non-Hispanic white, Chinese, African and Hispanic Americans, respectively.

[†] Mean ABI $\pm$ SD was 1.1 ± 0.1 for each of the four races/ethnicities considered ($P < 0.001$)

[‡] Mean carotid IMT (mm) $\pm$ SD was 0.71 ± 0.21 , 0.68 ± 0.19 , 0.75 ± 0.21 , 0.69 ± 0.19 ($P < 0.001$) for non-Hispanic white, Chinese, African and Hispanic Americans, respectively.