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To cite this article: Andrea Baccarelli, Letizia Tarantini, Robert O. Wright, Valentina Bollati, Augusto A. Litonjua, Antonella Zanobetti, David Sparrow, Pantel S. Vokonas & Joel Schwartz (2010) Repetitive element DNA methylation and circulating endothelial and inflammation markers in the VA normative aging study, *Epigenetics*, 5:3, 222-228, DOI: [10.4161/epi.5.3.11377](https://doi.org/10.4161/epi.5.3.11377)

To link to this article: <http://dx.doi.org/10.4161/epi.5.3.11377>



Published online: 01 Apr 2010.



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Repetitive element DNA methylation and circulating endothelial and inflammation markers in the VA normative aging study

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Key words: cell adhesion molecules, epidemiology, cardiovascular diseases, risk factors, LINE-1, VCAM-1

Background: Lower blood DNA methylation has been associated with atherosclerosis and high cardiovascular risk. Mechanisms linking DNA hypomethylation to increased cardiovascular risk are still largely unknown.

In a population of community-dwelling elderly individuals, we evaluated whether DNA methylation in LINE-1 repetitive element, heavily methylated sequences dispersed throughout the human genome, was associated with circulating Vascular Cell Adhesion Molecule-1 (VCAM-1), Inter-Cellular Adhesion Molecule-1 (ICAM-1) and C-reactive protein (CRP).

Methods and results: We measured LINE-1 methylation by bisulfite PCR-Pyrosequencing on 742 blood DNA samples from male participants in the Boston area Normative Aging Study (mean age = 74.8 years). Mean serum VCAM-1 increased progressively in association with LINE-1 hypomethylation (from 975.2 to 1063.4 ng/ml in the highest vs. lowest methylation quintiles; p trend = 0.004). The association between VCAM-1 and LINE-1 hypomethylation was significant in individuals without ischemic heart disease or stroke ($n = 480$; $p = 0.001$), but not in those with prevalent disease ($n = 262$; $p = 0.57$). Serum ICAM-1 and CRP were not associated with LINE-1 methylation (p trend = >0.25). All results were confirmed by multivariable analyses adjusting for age, BMI, smoking, pack-years and ischemic heart disease/stroke.

Conclusions: LINE-1 element hypomethylation is associated with higher serum VCAM-1. Our data provide new insights into epigenetic events that may accompany the development of cardiovascular disease.

Introduction

DNA methylation is a reversible epigenetic mechanism that, in mammals, modifies genome function through the addition of methyl groups to cytosine to form 5-methyl-cytosine (5mC).¹ About 55% of the human genome consists of repetitive elements, including approximately 500,000 Long Interspersed Nucleotide Elements (LINE-1), which are heavily methylated.² Demethylation of LINE-1 elements increases their activity as retrotransposable sequences, which may induce genomic alterations by insertion and/or homologous recombination.³ In addition, LINE-1 demethylation may increase transcription of genes that have LINE-1 sequences in their regulatory regions.⁴ Repetitive elements are globally activated during conditions of cellular stress,^{5,6} and LINE-1 expression has been recently identified as a mediator of ischemic heart damage.⁷ Because of their high representation throughout the genome, LINE-1 methylation has been shown to correlate with global genomic DNA methylation content⁸ and is used as a measure of global DNA methylation.²

DNA methylation changes have been suggested to play a role in cardiovascular disorders and atherosclerosis.⁹ Mutant mice with reduced global DNA methylation develop aortic lipid deposits that resemble atherosclerotic fatty streaks.¹⁰ In human¹¹ and animal studies,¹² lower global or LINE-1 DNA methylation has been observed in individuals with atherosclerotic disease both in atherosclerotic lesions and blood.¹³ Blood DNA hypomethylation has been also found in conditions at high risk of cardiovascular disease such as older age,^{14,15} smoking,¹⁶ exposure to air particulate pollution,¹⁷⁻¹⁹ folate deficiency,²⁰ hyperhomocysteinaemia¹³ and end-stage renal disease.²⁰ However, the mechanisms linking DNA hypomethylation to increased cardiovascular risk are still largely unknown. Understanding the pathways connecting potentially-modifiable epigenetic mechanisms, such DNA methylation,¹ to cardiovascular risk may give further insight in disease etiology and help develop novel preventive measures.

In keeping with growing biological evidence indicating that atherosclerosis is largely an inflammatory disease,²¹ circulating factors related to inflammation and inflammation-related endothelial dysfunction have been increasingly investigated as

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Submitted: 12/03/09; Accepted: 02/01/10

Previously published online: www.landesbioscience.com/journals/epigenetics/article/11377

Table 1. Characteristics of the study population [mean (min-max) or n (%)]

All visits, n = 742		
Age, years	73.8	(55.3–92.6)
Body Mass Index, kg/m ²	28.1	(19.1–52.6)
Smoking		
Never	235	(31.7%)
Ever	507	(68.3%)
Pack-years ^a	31.0	(0.1–131.3)
History of Ischemic Heart Disease or Stroke		
No	480	(64.7%)
Yes	262	(35.3%)
VCAM-1, ^b ng/ml	1022.9	(385.7–4031.3)
ICAM-1, ^b ng/ml	303.3	(39.0–1516.3)
CRP, ^b mg/l	1.8	(0.1–172.8)
LINE-1 methylation, %mC ^c	77.0	(66.2–86.1)

This table includes data collected at each visit for all measurements on all subjects. ^aMean and range (min-max) among ever smokers.

^bGeometric means are reported to account for lognormally distributed data. ^cPercentage of methylated cytosine.

predictors of cardiovascular disease in general populations. In particular, inflammatory or endothelial markers such as Vascular Cell Adhesion Molecule-1 (VCAM-1), Intracellular Adhesion Molecule-1 (ICAM-1) and C-Reactive Protein (CRP), have been identified as independent predictors of cardiovascular disease in human prospective studies.^{22–24} Inflammation involves recruitment of inflammatory cells from blood and their transendothelial migration mediated by adhesion molecules.²⁵ Consequently, cell adhesion molecules such as VCAM-1 and ICAM-1 are detected more commonly in human atherosclerotic lesions than in healthy arterial tissue.²² Although increased cell surface expression of these molecules is difficult to quantify *in vivo* at the site of the lesion, soluble forms that reflect such processes can be measured in serum or plasma.²⁶ In addition, several investigations have demonstrated that CRP is a risk factor for cardiovascular diseases.^{27–32} CRP is released in the systemic circulation acting as the final end product of various inflammatory pathways that converge to determine atherosclerosis and acute coronary syndromes. Thus, circulating CRP may reflect the cumulative effect of local vascular inflammation, as well as have direct proinflammatory or procoagulant effects on the endothelium.³³

To clarify the potential mechanisms linking DNA hypomethylation to cardiovascular disease, we evaluated whether DNA methylation in repetitive elements, such as LINE-1, that are widely represented across the human genome was associated with circulating VCAM-1, ICAM-1 and CRP in a population of community-dwelling elderly individuals in the greater Boston area.

Results

The characteristics of the study population are shown in **Table 1**. The study subjects were elderly individuals (mean age = 73.8 years, min 55.3, max 92.6). Ischemic heart disease or stroke were

found at 262 of the 742 visits (35.3%) that were included in the present analysis. Mean serum VCAM-1 was 1022.9 ng/ml (min 385.7–max 4031.3), mean serum ICAM-1 was 303.3 ng/ml (min 39.0–max 1516.3), and mean serum CRP was 1.8 mg/l (min 0.1–max 172.8). Mean blood DNA methylation of LINE-1 elements was 77.0 %mC (min 66.2–max 86.1).

VCAM-1 levels increased significantly with age ($p < 0.001$), whereas no age-related change in ICAM-1 and CRP was observed (**Table 2**). Higher body mass index was associated with lower levels of VCAM-1 ($p = 0.02$) and higher CRP ($p < 0.001$). Cumulative smoking, as reflected in smoking pack-years, showed associations with both ICAM-1 ($p = 0.001$) and CRP ($p < 0.001$), particularly due to increases in individuals with >26 pack-years. Individuals with ischemic heart disease or stroke exhibited higher serum levels of VCAM-1 ($p < 0.001$), ICAM-1 ($p < 0.001$) and CRP ($p = 0.02$).

Serum VCAM-1 increased progressively in association with LINE-1 hypomethylation (**Table 3**). In unadjusted models, mean VCAM-1 was 975.2 ng/ml (95% CI 926.2–1026.7) in the highest methylation quintile and increased up to 1063.4 ng/ml (95% CI 1010.1–1119.4) in the lowest methylation quintile ($p = 0.004$ for trend across quintiles). Models adjusted for age, body mass index, smoking (ever/never, pack-years), and ischemic heart disease or stroke confirmed the association between LINE-1 hypomethylation and serum VCAM-1 (**Table 3**). Adjusted mean VCAM-1 increased progressively from 974.7 ng/ml (95% CI 927.6–1024.1) in the highest methylation quintiles to 1060.0 ng/ml (95% CI 1009.2–1113.2) in the lowest methylation quintiles ($p = 0.01$ for trend across quintiles). ICAM-1 and CRP levels did not show any significant association with LINE-1 methylation ($p = >0.25$) [**Table 3**].

Table 4 shows the association of LINE-1 hypomethylation with serum VCAM-1, ICAM-1 or CRP stratified by prevalent ischemic heart disease or stroke at the examination. In subjects without ischemic heart disease or stroke, serum VCAM-1 increased progressively in association with LINE-1 hypomethylation. In unadjusted models, mean VCAM-1 was 921.6 ng/ml (95% CI 868.5–1098.0) in the highest methylation quintile and increased progressively up to 1030.5 ng/ml (95% CI 967.1–1098.0) in the lowest methylation quintiles ($p = 0.001$ for trend across quintiles). Models adjusted for age, body mass index and smoking (ever/never, pack-years) confirmed the association between LINE-1 hypomethylation and serum VCAM-1 among subjects free of ischemic heart disease or stroke (**Table 4**). In subjects with prevalent ischemic heart disease or stroke at the examination, we did not observe any association between serum VCAM-1 and LINE-1 hypomethylation (**Table 4**). ICAM-1 and CRP were not associated with LINE-1 hypomethylation, regardless of the presence of ischemic heart disease or stroke (**Table 4**).

Discussion

In this study on a population of elderly individuals, we found that hypomethylation of LINE-1 repetitive elements was associated with increasing circulating levels of VCAM-1, particularly

Table 2. Serum level of VCAM-1, ICAM-1 and CRP, by subjects' characteristics

	n	(%)	VCAM-1 (ng/ml)			ICAM-1 (ng/ml)			CRP (mg/l)		
			Mean ^a	(95% CI) ^a	p value ^b	Mean ^a	(95% CI) ^a	p-value ^b	Mean ^a	(95% CI) ^a	p-value ^b
Age, years											
55.3–69.4	184	(24.8%)	910.9	(869.7–954.0)		304.8	(293.2–316.8)		1.77	(1.51–2.08)	
69.5–74.1	187	(25.2%)	1010.1	(966.6–1055.5)		296.5	(285.9–307.4)		1.63	(1.40–1.91)	
74.2–78.9	185	(24.9%)	1048.5	(1002.8–1096.2)		304.3	(293.2–315.7)		1.95	(1.66–2.28)	
79.0–92.6	186	(25.1%)	1154.2	(1101.5–1209.4)	<0.001	309.8	(297.9–322.2)	0.64	1.85	(1.58–2.18)	0.37
Body Mass Index, kg/m²											
19.1–25.4	185	(24.9%)	1067.5	(1015.8–1121.8)		301.3	(289.3–313.8)		1.51	(1.29–1.77)	
25.5–27.4	186	(25.1%)	1056.5	(1007.8–1107.6)		302.4	(291.0–314.2)		1.55	(1.33–1.81)	
27.5–30.1	185	(24.9%)	1000.4	(953.5–1049.5)		303.1	(291.5–315.1)		1.75	(1.49–2.04)	
30.2–52.6	186	(25.1%)	986.9	(939.3–1036.8)	0.02	308.7	(296.5–321.3)	0.42	2.57	(2.19–3.01)	<0.001
Smoking											
Never	235	(31.7%)	1062.3	(1014.8–1112.0)		298.7	(287.7–310.1)		1.71	(1.48–1.98)	
Ever	507	(68.3%)	1011.4	(980.0–1043.8)	0.08	305.2	(297.4–313.2)	0.55	1.84	(1.67–2.03)	0.43
Pack-years											
0	235	(31.9%)	1062.3	(1014.8–1112.0)		298.7	(287.7–310.1)		1.71	(1.48–1.98)	
0.1–25	255	(34.6%)	994.8	(951.9–1039.6)		298.4	(287.9–309.4)		1.56	(1.36–1.79)	
26–131.3	248	(33.4%)	1034.4	(988.8–1082.0)	0.98	314.7	(303.4–326.5)	0.001	2.15	(1.87–2.48)	<0.001
Ischemic Heart Disease or Stroke											
No	480	(64.7%)	985.5	(954.9–1017.0)		294.7	(287.2–302.4)		1.67	(1.51–1.85)	
Yes	262	(35.3%)	1108.2	(1062.7–1155.6)	<0.001	321.1	(310.3–332.3)	<0.001	2.06	(1.79–2.35)	0.02

^aGeometric means and 95% confidence intervals computed from mixed regression model accounting for repeated measures. ^bTests for differences between categories (2-category variables) or for linear trend across categories (3+ category variables).

Table 3. Serum level of VCAM-1, ICAM-1 and CRP, by quintiles of global DNA methylation in LINE-1 elements

LINE-1 methylation	Mean ^a	(95% CI) ^a	p trend ^b	Adjusted by multiple covariates ^c		
				Unadjusted	Mean ^a	(95% CI) ^a
Serum level of Vascular Cell Adhesion Molecule-1 (VCAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	975.2	(926.2–1026.7)		974.7	(927.6–1024.1)	
77.5–78.4 %5-mC	1022.7	(974.0–1073.9)		1015.8	(968.9–1064.9)	
76.6–77.4 %5-mC	1023.9	(974.8–1075.6)		1024.6	(977.0–1074.4)	
75.4–76.5 %5-mC	1049.2	(997.4–1103.7)		1057.2	(1007.0–1109.8)	
66.2–75.3 %5-mC	1063.4	(1010.1–1119.4)	0.004	1060.0	(1009.2–1113.2)	0.01
Serum level of Inter Cellular Adhesion Molecule-1 (ICAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	298.6	(286.4–311.3)		298.2	(286.2–310.7)	
77.5–78.4 %5-mC	307.4	(295.5–319.7)		306.4	(294.7–318.5)	
76.6–77.4 %5-mC	307.8	(295.9–320.3)		308.3	(296.4–320.6)	
75.4–76.5 %5-mC	313.1	(300.6–326.2)		314.5	(302.1–327.5)	
66.2–75.3 %5-mC	292.9	(280.9–305.3)	0.76	292.1	(280.4–304.3)	0.69
Serum level of C-reactive protein (CRP, mg/l), by LINE-1 methylation						
78.4–86.1 %5-mC	2.05	(1.72–2.45)		2.05	(1.73–2.43)	
77.5–78.4 %5-mC	1.57	(1.32–1.87)		1.58	(1.33–1.86)	
76.6–77.4 %5-mC	1.72	(1.44–2.04)		1.73	(1.46–2.04)	
75.4–76.5 %5-mC	1.99	(1.67–2.37)		2.00	(1.69–2.37)	
66.2–75.3 %5-mC	1.70	(1.43–2.03)	0.25	1.70	(1.43–2.01)	0.30

^aGeometric means and 95% confidence intervals computed from mixed regression model accounting for repeated measures. ^bp value for the association of LINE-1 methylation with VCAM-1, ICAM-1 or CRP (Test for linear trend across categories of LINE-1 methylation). ^cResults from models adjusted by age, body mass index, smoking (never, former, current), pack-years of smoking, existing diagnosis of ischemic heart disease or stroke.

Table 4. Serum level of VCAM-1, ICAM-1 and CRP, by quintiles of global DNA methylation in LINE-1 elements in subjects free of cardiovascular disease or in subjects with existing diagnosis of cardiovascular disease

LINE-1 methylation	Mean ^a	(95% CI) ^a	p trend ^b	Mean ^a	(95% CI) ^a	p trend ^b
Unadjusted			Adjusted by multiple covariates ^c			
Subjects free of Ischemic Heart Disease or Stroke at the Examination (number of examinations = 480)						
Serum level of Vascular Cell Adhesion Molecule-1 (VCAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	921.6	(868.5–1098.0)		920.5	(868.5–975.7)	
77.5–78.4 %5-mC	984.3	(929.6–1088.5)		981.4	(927.9–1038.0)	
76.6–77.4 %5-mC	969.5	(913.8–1028.5)		966.5	(912.0–1024.2)	
75.4–76.5 %5-mC	1022.6	(960.7–1088.5)		1023.9	(963.3–1088.4)	
66.2–75.3 %5-mC	1030.5	(967.1–1098.0)	0.001	1031.5	(969.7–1097.2)	0.007
Serum level of Inter Cellular Adhesion Molecule-1 (ICAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	290.7	(276.9–305.3)		290.9	(277.0–305.4)	
77.5–78.4 %5-mC	294.8	(281.3–309.0)		294.5	(281.0–308.7)	
76.6–77.4 %5-mC	292.7	(278.8–307.3)		293.1	(279.2–307.7)	
75.4–76.5 %5-mC	307.9	(292.6–324.2)		308.1	(292.7–324.3)	
66.2–75.3 %5-mC	282.6	(268.3–297.6)	0.76	282.1	(267.9–297.0)	0.65
Serum level of C-reactive protein (CRP, mg/l), by LINE-1 methylation						
78.4–86.1 %5-mC	1.90	(1.54–2.34)		1.90	(1.55–2.33)	
77.5–78.4 %5-mC	1.53	(1.24–1.89)		1.54	(1.26–1.88)	
76.6–77.4 %5-mC	1.53	(1.24–1.90)		1.55	(1.26–1.90)	
75.4–76.5 %5-mC	1.94	(1.55–2.42)		1.94	(1.56–2.41)	
66.2–75.3 %5-mC	1.52	(1.22–1.89)	0.52	1.51	(1.22–1.87)	0.63
Subjects with Existing Diagnosis of Ischemic Heart Disease or Stroke at the Examination (number of examinations = 262)						
Serum level of Vascular Cell Adhesion Molecule-1 (VCAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	1084.8	(983.0–1197.0)		1088.6	(991.1–1195.7)	
77.5–78.4 %5-mC	1105.7	(1008.6–1212.1)		1096.9	(1003.4–1198.9)	
76.6–77.4 %5-mC	1115.8	(1022.3–1217.7)		1133.4	(1041.4–1233.5)	
75.4–76.5 %5-mC	1116.2	(1025.2–1215.3)		1123.6	(1035.8–1218.7)	
66.2–75.3 %5-mC	1115.6	(1024.3–1215.0)	0.57	1111.9	(1024.5–1206.6)	0.38
Serum level of Inter Cellular Adhesion Molecule-1 (ICAM-1, ng/ml), by LINE-1 methylation						
78.4–86.1 %5-mC	313.4	(289.8–339.0)		312.7	(289.2–338.2)	
77.5–78.4 %5-mC	335.2	(312.3–359.8)		333.5	(310.7–358.0)	
76.6–77.4 %5-mC	332.9	(311.2–356.1)		335.1	(313.2–358.6)	
75.4–76.5 %5-mC	321.7	(300.9–344.1)		322.0	(301.2–344.3)	
66.2–75.3 %5-mC	312.3	(291.9–334.1)	0.26	312.8	(292.4–334.6)	0.27
Serum level of C-reactive protein (CRP, mg/l), by LINE-1 methylation						
78.4–86.1 %5-mC	2.42	(1.75–3.34)		2.41	(1.76–3.29)	
77.5–78.4 %5-mC	1.64	(1.20–2.23)		1.67	(1.23–2.27)	
76.6–77.4 %5-mC	2.13	(1.59–2.86)		2.14	(1.60–2.85)	
75.4–76.5 %5-mC	2.09	(1.58–2.77)		2.10	(1.59–2.76)	
66.2–75.3 %5-mC	2.00	(1.51–2.66)	0.20	2.02	(1.53–2.66)	0.28

^aGeometric means and 95% confidence intervals from mixed regression model accounting for repeated measures. ^bp value for the association of LINE-1 methylation with VCAM-1, ICAM-1 or CRP (Test for linear trend across categories of LINE-1 methylation). ^cResults adjusted by age, body mass index, smoking (never, former, current) and pack-years of smoking.

in subjects free of cardiovascular disease at the examination. ICAM-1 and CRP levels did not show any significant association with LINE-1 methylation.

VCAM-1, ICAM-1 and CRP have been identified as independent markers of cardiovascular disease.²²⁻²⁴ VCAM-1 has been described as a mechanistically-related predictor of cardiovascular disease, because it is rapidly expressed in pro-atherosclerotic conditions and proven to play a critical biological role in several steps of atherosclerosis.^{37,38} Processes related to cardiovascular disease, such as oxidative stress,³⁹ atherosclerosis¹³ and aging,¹⁴ have been associated with lower DNA methylation content in blood DNA. In vascular tissues, hypomethylated DNA has been shown to be prone to mutations or aberrant gene expression patterns leading to the transition from normal phenotype to vascular fibrocellular lesions by increasing proliferation of vascular smooth cells and lipid deposition.¹³ Blood DNA methylation has been shown to undergo progressive changes as individuals age.^{14,15} Initial investigations have found global blood DNA hypomethylation in patients with atherosclerotic disease,¹³ but whether lower DNA methylation predicts future cardiovascular risk is undetermined.

In our study we found an association between LINE-1 hypomethylation and VCAM-1, but not with ICAM-1 and CRP. Using the Genomatix software, EIDorado annotation lists (GmbH, München, Germany) we analyzed the DNA sequence of the VCAM-1, ICAM-1 and CRP genes. We found that the VCAM-1 gene region contains eight LINE-1 repetitive elements, while ICAM-1 does not contain any LINE-1 sequence, and CRP contains only one. It is thus possible that diffuse hypomethylation of LINE-1 sequences may be associated with location-specific hypomethylation in the VCAM-1 region. As gene-specific hypomethylation usually results in higher gene expression,¹ lower methylation of LINE-1 sequences in the VCAM-1 regions may lead to VCAM-1 overexpression. However, because our approach for LINE-1 analysis was based on the amplification of a representative pool of LINE-1 elements as a global measure of genomic repetitive element methylation, it did not allow for the evaluation of individual loci. In addition, we measured LINE-1 methylation in blood peripheral leukocytes, and we cannot assume that leukocyte LINE-1 methylation reflects methylation in other tissues, such as vascular endothelial cells, that are the major source of VCAM-1 production.⁴⁰ On the other hand, LINE-1 methylation in blood leukocytes might represent a general status of cellular deficit of methyl donors in response to acquired risk factors, or other causes determining lower methylation.² Whether LINE-1 methylation in blood leukocytes correlates with levels in vascular tissues remains to be determined. Overall, the mechanism linking LINE-1 hypomethylation and VCAM-1 levels in our study are unclear, and the mechanisms for the observed association will need to be further investigated in future studies.

In our study, we observed a significant association between LINE-1 hypomethylation and VCAM-1 serum levels only in subjects without prevalent ischemic heart disease or stroke. Subjects with prevalent ischemic heart disease and stroke diagnosis did not show any association between VCAM-1 and LINE-1 hypomethylation perhaps because they already exhibited higher serum levels of VCAM-1 as a result of the disease. This finding

would suggest that the relation between LINE-1 hypomethylation and VCAM-1 may represent an early event in the etiology of cardiovascular disease. However, as the subgroup of subjects with ischemic heart disease and stroke was smaller than the subgroup without the diseases, we cannot exclude that the lack of association might have been determined by lower statistical power.

In our study, we measured DNA methylation in LINE-1 repeated sequence. These sequences are widespread across the human genome, are heavily methylated, and their methylation levels have been shown to correlate with global DNA methylation content.^{2,8}

We performed our study using Pyrosequencing, a quantitative analysis of DNA methylation which is highly reproducible and accurate at measuring DNA methylation. In addition, we repeated DNA methylation analysis three times on each sample and used the replicate average to minimize the assay variability.

Our study had the advantage of being based on a population of aging individuals unselected on the basis of disease status. However, our results can only be generalized to an aged population that consists of older males who are almost all white. Our findings should be confirmed in future studies including women, as well as different age and ethnic groups. As we measured methylation in blood DNA, our results might have reflected shifts in the proportions of white-blood-cell subsets. However, adjustment in multivariable analysis for percent neutrophils and lymphocytes, the major white blood cell types, did not affect the results.

The increase in VCAM-1 we observed in association with LINE-1 hypomethylation was relatively small. Therefore, our results should be interpreted with caution, in particular with respect to any inference on clinical risk.

In conclusion, our results on an elderly population in the Boston area demonstrated for the first time an association between lower blood repetitive element methylation and VCAM-1 expression. Our data provide new insights into epigenetic events that may accompany the development of cardiovascular diseases. Whether LINE-1 hypomethylation, either across the genome or in the VCAM-1 region, predicts the risk of cardiovascular disease should be addressed in future longitudinal studies.

Methods

Study subjects. Our study population consisted of 593 white males, evaluated between May 2000 and July 2007, as part of the Normative Aging Study (NAS), a longitudinal study of aging established in 1963 by the U.S. Veterans Administration.³⁴ The NAS participants are recalled for examination every 3–5 years and at each visit all study subjects are asked to donate a 7 ml blood sample. Of all the 593 study subjects, 444 had blood DNA methylation, VCAM-1, ICAM-1 and CRP measured in only one blood sample, and 149 in two blood samples taken at different visits, for a total of 742 samples. At each visit, all subjects were assessed for cardiovascular disease, including nonfatal ischemic heart disease or stroke, based on physician examination, electrocardiography and/or medical records.³⁵ This study was

approved by the Institutional Review Boards of all participating Institutions and all participants gave written informed consent to the study.

Methylation analysis. DNA was extracted from stored frozen buffy coat of 7 mL whole blood, using the QiAmp DNA blood kits (QIAGEN, Hilden, Germany). 500 ng DNA (concentration 50 ng/μl) was treated using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 μl of M-Elution Buffer.

Analysis of repetitive element DNA methylation was performed using previously published methods.^{2,36} PCR primers were designed towards a consensus LINE-1 sequence and allowed the amplification of a representative pool of repetitive elements to serve as a surrogate for global DNA methylation changes. The degree of methylation was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC) measured in each individual sample. We used built-in controls to verify bisulfite conversion efficiency. Every sample was tested three times for each marker to confirm reproducibility and increase precision of our results. The average of the three replicates was used in statistical analyses.

VCAM-1, ICAM-1 and CRP measurement. VCAM-1, and ICAM-1 were measured in serum using the enzyme-linked immunosorbent assay method (R&D Systems, Minneapolis, MN). Sensitivity of the assay for VCAM-1 was 2.0 ng/mL and day-to-day variabilities of the assay at concentrations of 9.8, 24.9 and 49.6 ng/mL were 10.2, 8.5 and 8.9%, respectively. Sensitivity of the assay for ICAM-1 was 0.35 ng/mL and the day-to-day variabilities of the assay at concentrations of 64.2, 117, 290 and 453 ng/mL were 10.1, 7.4, 6.0 and 6.1%, respectively. Serum CRP was measured using immunoturbidimetric assay on the Hitachi 917 analyzer (Roche Diagnostics-Indianapolis, IN) with reagents and calibrators from Denka Seiken (Niigata, Japan).³² Technicians were blinded to LINE-1 methylation and all other participant data.

Statistical methods. Because our study included repeated measures of VCAM-1, ICAM-1, CRP and DNA methylation for many participants, the data are correlated. To deal with this, we fitted mixed effects models (xtmixed in Stata 9.0/SE) assuming:

$$Y_{it} = b_0 + u_i + b_1 X_{lit} + \dots + b_p X_{pit} + \beta \text{Methylation}_i + \varepsilon_{it}$$

where Y_{it} is the level of VCAM-1, ICAM-1 or CRP in subject i at time t , b_0 is the overall intercept, u_i is the separate random intercept for subject i , and X_{lit} - X_{pit} are the p covariates for subject i at each time t . VCAM-1, ICAM-1 and CRP were log-transformed to approximate normal distribution. We evaluated the association of with VCAM-1, ICAM-1 or CRP with LINE-1 methylation in unadjusted models, as well as in multivariable models including as covariates age, body mass index, cigarette smoking (never, former, current), pack-years of smoking and existing diagnosis of ischemic heart disease or stroke. In these models, LINE-1 methylation was used as a categorical variable based on quintiles. From these models, we calculated the unadjusted and covariate-adjusted geometric means of VCAM-1, ICAM-1 and CRP for each category of LINE-1 methylation using the post-estimation command `adjust` in Stata 9.0/SE. Tests for trend across categories of LINE-1 methylation were computed in the same modeling framework by assigning a score between 1 and 5 to each category of LINE-1 methylation. Using the same statistical approach, we evaluated the associations of VCAM-1, ICAM-1 or CRP with the general characteristics of the subjects, including age, body mass index, smoking, pack years and existing diagnosis of ischemic heart disease or stroke. As a sensitivity analysis, we fit all multivariable models after including as independent variables, in addition to the covariates listed above, the percent lymphocytes and neutrophils in the differential blood counts. The results from this set of models were similar to those obtained from the main analysis, with no change in statistical significance. All tests were two-tailed, $p < 0.05$ were considered statistically significant. All analyses were performed in Stata 9.0/SE (Stata Corp., College Station, TX).

Acknowledgements

This work was supported by funding from National Institute of Environmental Health Sciences (NIEHS) ES015172, ES00002; Environmental Protection Agency (EPA) R83241601 and R827353; CARIPLO Foundation 2007-5469; and PRIN 2007 program. The VA Normative Aging Study is supported by the Cooperative Studies Program/Epidemiology Research and Information Center of the U.S. Department of Veterans Affairs and is a component of the Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC).

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