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Increased Homocysteine and *S*-Adenosylhomocysteine Concentrations and DNA Hypomethylation in Vascular Disease

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Background: **The pathogenic mechanism of homocysteine's effect on cardiovascular risk is poorly understood. Recent studies show that DNA hypomethylation induced by increases in *S*-adenosylhomocysteine (AdoHcy), an intermediate of Hcy metabolism and a potent inhibitor of methyltransferases, may be involved in homocysteine-related pathology.**

Methods: **We measured fasting plasma total Hcy (tHcy), AdoHcy, and *S*-adenosylmethionine (AdoMet) and methylation in leukocytes in 17 patients with vascular disease and in 15 healthy, age- and sex-matched controls.**

Results: **Patient with vascular disease had significantly higher plasma tHcy and AdoHcy concentrations and significantly lower plasma AdoMet/AdoHcy ratios and genomic DNA methylation. AdoMet concentrations were not significantly different between the two groups. More than 50% of the patients fell into the highest quartiles of plasma tHcy, AdoHcy, and [^{3}H]dCTP incorporation/μg of DNA (meaning the lowest quartile of DNA methylation status) and into the lowest quartile of the AdoMet/AdoHcy ratios of the control group. Plasma tHcy was significantly correlated with plasma AdoHcy and AdoMet/AdoHcy ratios (n = 32; $P < 0.001$). DNA methylation status was significantly correlated with plasma tHcy and AdoHcy (n = 32; $P < 0.01$) but not with plasma AdoMet/AdoHcy ratios.**

Conclusion: **Global DNA methylation may be altered in vascular disease, with a concomitant increase in plasma tHcy and AdoHcy.**

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DNA methylation is an important epigenetic feature of DNA that plays a critical role in gene regulation *(1)* and has recently been implicated in chronic disease states, including atherosclerosis *(2, 3)*. DNA methylation patterns are maintained by DNA methyltransferases *(4–7)*, which use *S*-adenosylmethione (AdoMet)[5] as the methyl group donor. AdoMet is also the methyl donor in other cellular methyltransferase reactions, including in RNA and protein methylation, synthesis, and detoxification processes. As a result of the transfer of the methyl group, AdoMet is converted to *S*-adenosylhomocysteine (AdoHcy) and a decrease in the AdoMet/AdoHcy ratio is often taken as an indicator of reduced cellular methylation capacity *(8–10)*. Under physiologic conditions, AdoHcy is hydrolyzed to Hcy and adenosine. This reaction is reversible, with a dynamic equilibrium that strongly favors AdoHcy synthesis rather than hydrolysis *(8)*. For this reason, the removal of Hcy and adenosine is crucial, and disturbed metabolic pathways that lead to increased intracellular Hcy concentrations will also increase AdoHcy *(10)*. AdoHcy binds to methyltransferases with higher affinity than does AdoMet, and as a potent inhibitor of most methylation reactions, including those of DNA methyltransferases, it potentially affects cell homeostasis *(11)*.

Hyperhomocysteinemia is a well-established risk fac-

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[5] Nonstandard abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; and tHcy, total homocysteine.

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tor for vascular disease, but the underlying pathogenic mechanism remains poorly understood, although thoroughly studied. Most studies have been focused on the direct effect of Hcy, but it was recently suggested that a chronic increase in AdoHcy (as a result of the Hcy-mediated reversal of the AdoHcy hydrolase reaction) might have significant pathologic consequences *(12)*. Furthermore, the role of epigenetic alterations has emerged as a crucial mechanism for regulating genes responsible for cell proliferation in atherosclerosis *(3)*. The present study was undertaken to assess the possible association between increased Hcy and epigenetic alterations in vascular disease. Plasma total homocysteine (tHcy), AdoMet, and AdoHcy concentrations, as well as leukocyte DNA methylation status, were evaluated in vascular disease and control groups. The results support the hypothesis that the pathogenic role of hyperhomocysteinemia in vascular disease might be mediated by AdoHcy accumulation and DNA hypomethylation.

Materials and Methods

PARTICIPANTS AND SAMPLE COLLECTION

We studied 17 male atherosclerotic vascular patients [mean (SD) age, 54 (5.8) years] and 15 healthy male controls [mean (SD) age, 53 (6.9) years]. Cases were recruited among patients who had been admitted to hospital with a diagnosis of stroke (n = 8) or angiographically confirmed myocardial infarction (n = 9); samples were collected at least 3 months after the occurrence. Controls were selected among hospital employees whose lifestyle details (i.e., smoking, alcohol consumption, medication, physical exercise, and personal and family histories) and baseline laboratory measurements were established by use of standardized questionnaires and protocols. The criteria for inclusion in the control group were: normal hematology and liver/renal function tests and no history of vascular pathology. Exclusion criteria for both groups were metabolic, hepatic, or renal pathology; cancer; alcohol or drug abuse; and use of vitamin B_6, vitamin B_{12}, or folic acid supplements within the 2 previous months. Written informed consent was obtained from all participants, and the Local Ethical Committee approved the study.

Blood samples were collected from all participants after overnight fasting by venipuncture into EDTA-containing tubes. One tube was immediately placed on ice and centrifuged at 4000*g* for 10 min at 4 °C. One plasma aliquot was stored at −20 °C until tHcy quantification, whereas 1000 μL was immediately deproteinized by adding 624 μL of 100 g/L perchloric acid and then stored at −20 °C until AdoMet and AdoHcy determinations. Hematologic analysis revealed no significant differences in the mononuclear vs polymorphonuclear cells ratios between the individuals of both groups. Genomic DNA was extracted from white blood cells according to standard phenol–chloroform procedures *(13, 14)* and then stored at 4 °C until quantification of global DNA methylation.

tHcy, AdoMet, AND AdoHcy MEASUREMENTS

Plasma tHcy was quantified in the thawed aliquots by a specific immunoassay (IMx; Abbott Laboratories).

For plasma AdoMet and AdoHcy quantification, the deproteinized samples were thawed and centrifuged, and the obtained supernatant was analyzed by stable-isotope-dilution tandem mass spectrometry, as described previously in detail *(15)*.

GLOBAL DNA METHYLATION STATUS

The global DNA methylation status was assessed by use of the cytosine extension assay *(16)*. Briefly, 1 μg of genomic DNA was digested overnight with a methylation-sensitive endonuclease (*Hpa*II), which left a single guanine overhang at unmethylated CpG sites. A second DNA aliquot was incubated without *Hpa*II and served as background control. A single nucleotide extension with [^{3}H]dCTP (Amersham Biosciences) was then performed. Duplicate 10-μL aliquots from each reaction were applied on Whatman DE-81 ion-exchange filters, dried, and washed three times with sodium phosphate buffer (pH 7.0) at room temperature. Filters were dried and processed for scintillation counting. Background radiolabel incorporation was subtracted from enzyme-treated samples, and the results were expressed as relative [^{3}H]dCTP incorporation/μg of DNA. Incorporation of the radiolabel was considered proportional to the number of unmethylated (cleaved) sites in DNA. All samples were analyzed at the same time to minimize variation. The within-assay imprecision of the cytosine extension assay was <6%.

STATISTICAL ANALYSIS

Data are presented as medians and interquartile ranges. The distributions of plasma tHcy, AdoMet, and AdoHcy and the relative [^{3}H]dCTP incorporation/μg of DNA were positively skewed and logarithmically transformed before further statistical analysis. Differences between patient and control groups were evaluated with the independent-samples Student *t*-test. Correlations were determined with the Spearman method; *P* values were two-tailed. Statistical significance was set at $P < 0.05$.

Results

The median plasma tHcy, AdoMet, and AdoHcy concentrations, the AdoMet/AdoHcy ratios, and the global DNA methylation status in the studied population are presented in Table 1. Compared with controls, patients had significantly higher ($P < 0.01$) plasma tHcy and AdoHcy concentrations, decreased ($P < 0.01$) plasma AdoMet/AdoHcy ratios, and lower ($P < 0.05$) global DNA methylation status. We observed no significant differences in plasma AdoMet concentrations between the two groups. The variables displaying statistical difference were subdivided into quartiles, and the patients and controls were distributed accordingly, as shown in Fig. 1. More than 50% of the patients fell into the highest quartiles of plasma tHcy and AdoHcy concentrations and [^{3}H]dCTP incorpo-

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Table 1. Plasma tHcy, AdoMet, and AdoHcy concentrations and global DNA methylation status in the studied population.[a]

	Patients (n = 17)	Controls (n = 15)
tHcy, μmol/L	10.4 (8.8–12.7)[b]	7.5 (6.5–8.9)
AdoMet, nmol/L	81.1 (63.0–107)	77.5 (67.0–96.0)
AdoHcy, nmol/L	16.9 (12.7–19.6)[b]	12.2 (10.2–16.9)
AdoMet/AdoHcy	4.8 (4.3–6.2)[b]	5.8 (5.3–6.9)
DNA methylation, dpm/μg[d] of DNA	14 900 (9993–19 130)[c]	10 155 (6843–11 518)

[a] Values are medians and interquartile ranges.
[b,c] Statistically different (Student *t*-test) between patients and controls: [b] $P < 0.01$; [c] $P < 0.05$.
[d] dpm, disintegrations per minute.

ration/μg of DNA (i.e., the lowest quartile of DNA methylation status) and into the lowest quartile of plasma AdoMet/AdoHcy ratios for the controls. When we considered the patients and controls together, we observed that the increase in plasma tHcy was significantly correlated with a parallel increase in plasma AdoHcy ($r = 0.81$; $P < 0.0001$) and with a decrease in plasma AdoMet/AdoHcy ratios ($r = -0.68$; $P < 0.0001$; Fig. 2).

We found no apparent association between plasma tHcy and AdoMet concentrations (data not shown). In addition, there was a significant correlation between DNA methylation status and plasma tHcy ($r = 0.47$, $P < 0.01$) and plasma AdoHcy concentrations ($r = 0.54$; $P < 0.01$; Fig. 3), but no correlation with plasma AdoMet/AdoHcy ratios (data not shown).

Discussion

A growing body of evidence has documented the role of hyperhomocysteinemia as an independent vascular risk factor. However, the mechanisms through which increased plasma Hcy concentrations cause endothelial dysfunction and atherosclerosis remain elusive. At present, atherogenesis is believed to involve mainly gene function, such as epigenetic changes leading to alterations in gene expression and genomic integrity *(2)*. Methylation is an

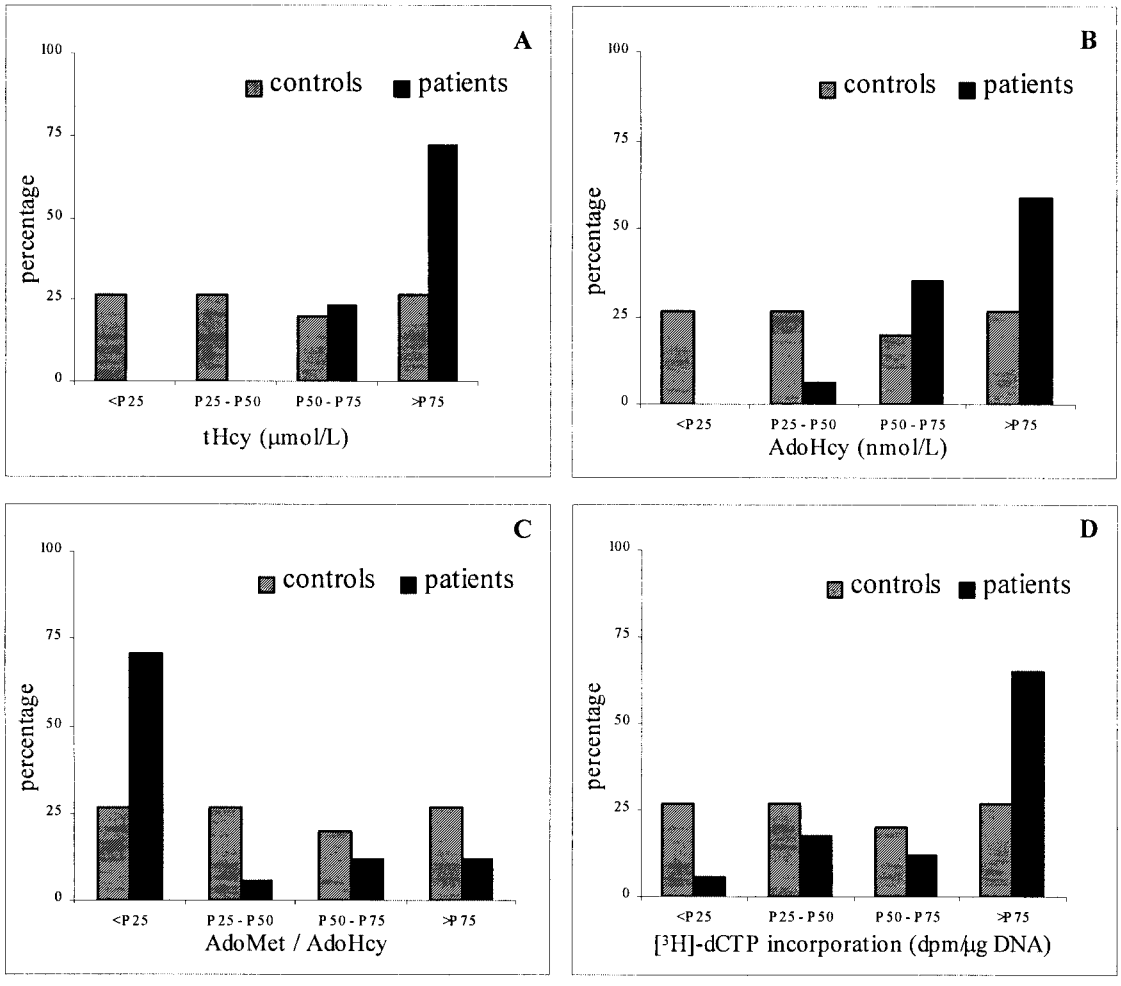

Fig. 1. Plasma tHcy (*A*) and AdoHcy (*B*) concentrations, AdoMet/AdoHcy ratios (*C*), and DNA methylation status (*D*) divided into quartiles and presented as percentages of the total number of studied controls (▨) and patients with vascular disease (■) included in each group.

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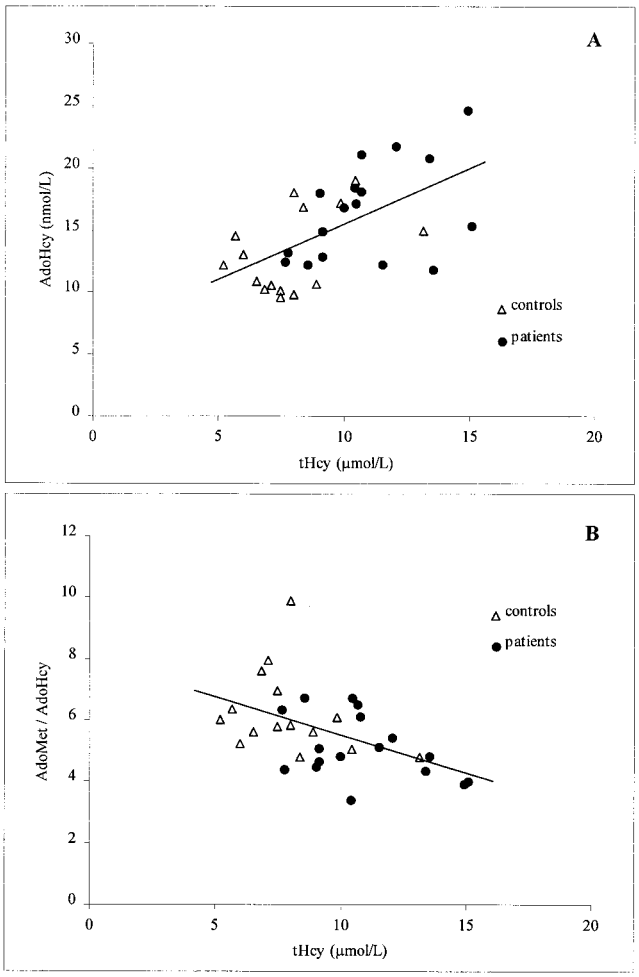

Fig. 2. Plots of individual values for plasma tHcy vs AdoHcy ($r = 0.81$; $P < 0.0001$; *A*) and for plasma tHcy vs AdoMet/AdoHcy ratio ($r = -0.68$; $P < 0.0001$; *B*) for each participant.

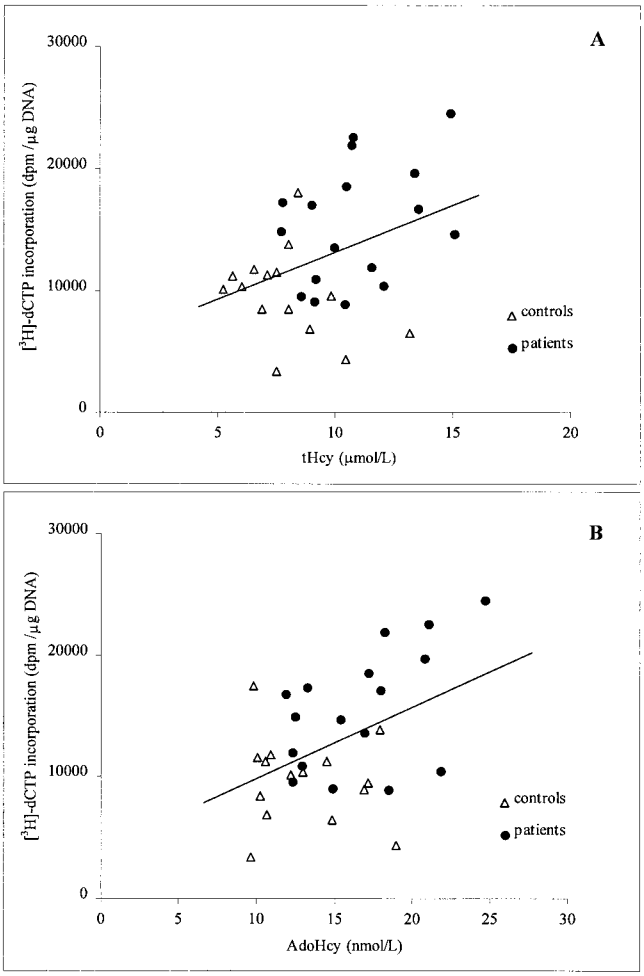

Fig. 3. Plots of individual values for plasma tHcy vs global DNA methylation status ($r = 0.47$; $P < 0.01$; *A*) and for plasma AdoHcy vs global DNA methylation status ($r = 0.54$; $P < 0.01$; *B*) for each participant.

important epigenetic feature of DNA, which is catalyzed by DNA methyltransferases with AdoMet as a methyl donor. Recently, it has been suggested that the pivotal biomarker of the association between hyperhomocysteinemia and vascular disease would not be Hcy itself, but increases in AdoHcy, secondary to the Hcy-mediated reversal of the AdoHcy hydrolase reaction. An increase in AdoHcy causes feedback inhibition of AdoMet-dependent methyltransferases, including the DNA methyltransferases *(12, 17)*. The tissue-specific metabolic pathways are crucial in determining the relative sensitivity to alterations in AdoHcy concentrations *(18)*. The demonstrated lack of cystathionine β-synthase *(19)* and betaine-homocysteine methyltransferase *(17)* activity in endothelial cells may enhance their sensitivity to AdoHcy accumulation and, consequently, to DNA hypomethylation. Our findings are in line with the hypothesis that the pathogenic role of hyperhomocysteinemia in vascular disease might be caused by AdoHcy accumulation and DNA hypomethylation. However, because of the small size of the study sample, further larger studies are needed to confirm these findings.

In the present report, patients with vascular disease had significantly higher plasma tHcy and AdoHcy concentrations, decreased plasma AdoMet/AdoHcy ratios, and lower global DNA methylation status compared with controls. Significantly, >50% of the patients fell into the highest quartiles for plasma tHcy and AdoHcy concentrations and into the lowest quartiles for plasma AdoMet/AdoHcy ratios and DNA methylation status for the controls (Fig. 1). In addition, when patients and controls were considered together, the increase in plasma tHcy was highly correlated with a parallel increase in plasma AdoHcy (Fig. 2A) and with a decrease in plasma AdoMet/AdoHcy ratios (Fig. 2B), but not with plasma AdoMet. Moreover, plasma tHcy and AdoHcy were significantly correlated with the global DNA methylation status (Fig. 3). However, we observed no correlation between plasma AdoMet concentrations or AdoMet/AdoHcy ratios and the global DNA methylation status.

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Similar observations have also been reported by Yi et al. *(20)*.

The intracellular AdoMet/AdoHcy ratio has been used as a predictor of cellular methylation capacity. A significant correlation between plasma and lymphocyte AdoMet/AdoHcy ratios has been reported *(12)*. This observation suggests that the plasma AdoMet/AdoHcy ratio may be used as a reasonable predictor of the cellular methylation status. In the present study, the lack of correlation between the plasma AdoMet/AdoHcy ratio and DNA methylation status could seem unexpected. However, the authors of two recent studies in humans *(20)* and animals *(18)* claimed that intracellular AdoHcy is a more reliable biomarker for cellular methylation status. Our findings are in accordance with this observation.

In conclusion, the present study shows that patients with vascular disease have disturbed global DNA methylation status associated with increased plasma tHcy and AdoHcy concentrations. Whether these observations contribute to the pathogenic role of hyperhomocysteinemia in vascular disease needs to be further ascertained.

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