

# Plasma S-Adenosylhomocysteine Is a Better Biomarker of Atherosclerosis Than Homocysteine in Apolipoprotein E-Deficient Mice Fed High Dietary Methionine<sup>1,2</sup>

Chi Liu, Qing Wang, Honghui Guo, Min Xia, Qin Yuan, Yan Hu, Huilian Zhu, Mengjun Hou, Jing Ma, Zhihong Tang, and Wenhua Ling\*

Department of Nutrition, School of Public Health, Sun Yat-sen University (Northern Campus), Guangzhou 510080, P. R. China

## Abstract

Homocysteine (Hcy) and S-adenosylhomocysteine (AdoHcy) are critical intermediates of methionine metabolism. To investigate which, if either, of these compounds is more closely related to atherosclerosis, we fed 5 groups of apolipoprotein E (apoE)-deficient mice different diets for 8 wk to induce changes in their plasma Hcy and AdoHcy concentrations. These included an AIN-93G control diet (C), this C diet supplemented with methionine (M), the M diet deficient in folates, vitamin B-6, and vitamin B-12 (M-V), this M diet supplemented with these B vitamins (M+V), and a C diet deficient in B vitamins (C-V). Compared with controls, mice fed the C-V diet had a moderate elevation in their plasma total Hcy (tHcy) levels; however, their plasma AdoHcy concentration and atherosclerotic lesion areas were not different. In contrast, the mice fed the M+V diet had larger atherosclerotic lesion areas and elevated plasma AdoHcy concentrations but their plasma tHcy concentration did not differ from that of the group C mice. The plasma AdoHcy concentration and aortic sinus lesion areas were positively correlated ( $r = 0.866$ ;  $P < 0.001$ ). We observed a negative correlation between the plasma AdoHcy concentration and both the DNA methyltransferase activity ( $r = -0.792$ ;  $P < 0.001$ ) and global DNA methylation status ( $r = -0.824$ ;  $P < 0.001$ ) in the aortic tissue. Hence, our study suggests that plasma AdoHcy is a better biomarker of atherosclerosis than Hcy and may accelerate the development of atherosclerotic lesions in apoE-deficient mice that have been fed a high methionine diet. The mechanisms underlying this effect may be related to the AdoHcy-mediated inhibition of DNA methylation in the aortic tissue. *J. Nutr.* 138: 311–315, 2008.

## Introduction

Homocysteine (Hcy)<sup>3</sup> is a sulfur-containing amino acid that functions as an intermediate in methionine metabolism. The hypothesis that Hcy causes vascular diseases was first proposed by McCully in 1969 (1) and numerous epidemiological studies have since demonstrated that an increased concentration of total Hcy (tHcy) in plasma is an independent risk factor for atherothrombotic diseases (2–4). However, the mechanisms underlying the onset of hyperhomocysteinemia related to atherogenesis remain largely unknown and it is still unclear whether a

normalization of the elevated plasma tHcy levels can reduce the onset of cardiovascular disease (5,6).

A recent study by Troen et al. (7) has shown that the extent of atherosclerosis development is independent of the plasma tHcy concentration in apolipoprotein E (apoE)-deficient mice fed with methionine-enriched and B vitamin-deficient or -supplemented diets. This finding suggested that the atherogenesis induced by a high dietary intake of methionine involves complex pathogenic mechanisms and that plasma Hcy may be an indirect indicator of atherosclerotic lesions. More attention has recently been paid, however, to another metabolic intermediate of methionine, S-adenosylhomocysteine (AdoHcy), which is the sole metabolic precursor of Hcy in a reversible reaction catalyzed by AdoHcy hydrolase. In a recent case-control study, Kerins et al. (8) demonstrated that an elevation in the plasma AdoHcy concentration is a much more sensitive indicator of cardiovascular disease than elevations in the plasma Hcy levels. This suggested that the plasma AdoHcy concentration may be a better biomarker of atherosclerosis than Hcy. Additional studies will be needed in the future, however, to more precisely determine the role of AdoHcy in atherosclerosis.

Atherogenesis is thought to involve alterations in gene expression and the corresponding activities of their gene products.

<sup>1</sup> Supported by a grant from the National Natural Science Foundation of China (30571568).

<sup>2</sup> Author disclosures: C. Liu, Q. Wang, H. Guo, M. Xia, Q. Yuan, Y. Hu, H. Zhu, M. Hou, J. Ma, Z. Tang, and W. Ling, no conflicts of interest.

<sup>3</sup> Abbreviations used: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; apoE, apolipoprotein E; C, AIN-93G diet; C-V, AIN-93G diet deficient in B vitamins; Dnmt, DNA methyltransferase; Hcy, homocysteine; M, AIN-93G diet supplemented with methionine; M+V, AIN-93G diet supplemented with methionine and B vitamins; M-V, AIN-93G diet supplemented with methionine but deficient in B vitamins; tHcy, total homocysteine.

\* To whom correspondence should be addressed. E-mail: lingwh@mail.sysu.edu.cn.

Epigenetic changes, particularly aberrant DNA methylation, serve as an important mechanism that controls gene expression in a variety of chronic diseases. In this regard, AdoHcy is a potential inhibitor of DNA methyltransferase (Dnmt), which could thus affect the DNA methylation pattern. In accordance with this notion, 2 independent studies of patients with vascular disease have previously shown that the concentration of Hcy increases in parallel with AdoHcy in plasma and that these increases were associated with lymphocyte DNA hypomethylation (9,10). Hence, it appears that an AdoHcy-mediated inhibition of DNA methylation may have a profound impact on the pathogenesis of atherosclerosis. However, the effects of AdoHcy upon the methylation of specific genes related to atherosclerosis, or across the whole genome, have not been documented thus far.

Our study was designed to examine the effects of an elevated plasma AdoHcy concentration on atherosclerotic lesions in apoE-deficient mice fed high levels of dietary methionine and to determine whether AdoHcy is a better biomarker of atherosclerosis than Hcy. We further investigated the potential relationship between the plasma AdoHcy concentration and the methylation of aortic tissue DNA during the development of atherosclerosis in the same mouse model.

## Materials and Methods

**Mice and diets.** Three-week-old male weanling apoE-deficient mice, which were purchased from Jackson Laboratories and maintained in our animal facility, were fed with a laboratory diet based on the AIN-93G formula (11). At 8 wk of age, these mice were divided into 5 groups of 10 that were matched for body weight. Each group received 1 of the following diets: an AIN-93G diet (C); the C diet supplemented with methionine (M); the M diet deficient in folate, vitamin B-6, and vitamin B-12 (M-V); the M diet supplemented with these B vitamins (M+V); and the C diet deficient in B vitamins (C-V). These experimental diets were modified according to Troen et al. (7) using vitamin-free casein and other vitamin mixtures (Harlan TEKLAD) to ensure variety in the vitamin intake of the mice. The nutrients contained in the diet of each group are listed in Table 1. All of the mice were then fed these experimental feeds for an additional 8 wk and had free access to both food and water. We weighed the mice every week and calculated the consumption of water and feed for each group. This study and all the procedures herein were approved by the Animal Care and Use Committee of Sun Yat-sen University.

**Tissue preparation and atherosclerotic lesion analysis.** After 8 wk of dietary intervention, the mice were food-deprived overnight and killed by exsanguination from the retro-orbital plexus. The hearts were then harvested intact and stored in 10% formalin buffer solution at 4°C. The whole length of the aorta was immediately snap-frozen in liquid nitrogen and stored at -80°C. Consecutive sections (8  $\mu$ m) were cut through the aortic sinus where the valve cusp was visible and then stained with oil red O and counterstained with hematoxylin. The lesion area was measured by means of direct image capture from an RGB camera (JVCky-F 30B)

**TABLE 1** Methionine, folate, vitamin B-6, and vitamin B-12 contents in the experimental diets used in this study

Group	L-Methionine	Folate	Vitamin B-6	Vitamin B-12
	g/kg	mg/kg	mg/kg	$\mu$ g/kg
C	—	2	7	25
M	10	2	7	25
M-V	10	0.11	0.15	1.8
M+V	10	10	35	125
C-V	—	0.11	0.15	1.8

and quantified using Optimas 4.1 software (12). The detectable atherosclerotic lesion areas were expressed as the percentage of the total cross-sectional vessel wall area and the mean lesion size from 5 sections was calculated for each mouse.

**Serum vitamin B-6, vitamin B-12, and folate.** Blood samples from the retro-orbital venous plexus were centrifuged at 1500  $\times$  g; 15 min to obtain serum, which was then stored at -20°C for subsequent testing. The serum pyridoxal phosphate (vitamin B-6) concentration was determined by HPLC (13) (510; Waters). The levels of serum vitamin B-12 and folate were measured using a chemiluminescence immunoassay (ACS 180; Bayer).

**Plasma tHcy and AdoHcy measurements.** The blood samples used to measure the plasma tHcy and AdoHcy concentrations were collected in EDTA-containing tubes, which were centrifuged at 3000  $\times$  g; 10 min within 30 min and stored at -20°C. Plasma tHcy concentrations were quantified using a fluorescence polarization immunoassay (Abbott IMx). For the estimation of the plasma AdoHcy concentration, we have developed a new method that is less time consuming than chromatographic separation. The principle of this technique is based on the competitive immunoassay using a monoclonal anti-AdoHcy antibody (14). Briefly, thawed plasma samples were deproteinized by adding a 1/11 volume of 100% trichloroacetic acid. After incubation on ice for 30 min, supernatants were obtained by centrifugation at 18,000 g; 15 min at 4°C and transferred into the wells of an AdoHcy-coated microtiter plate. The chemicals utilized for the subsequent competitive immunoassay were purchased from Axis-Shield. We defined the quantification limit as 5 nmol/L and the reproducibility was consistent. Eight control standards for calibration were prepared by the addition of AdoHcy into control plasma at concentrations ranging from 5 to 200 nmol/L and then subjecting these controls to the same pretreatment procedures as the unknown samples to construct a calibration curve. A total of 200  $\mu$ L of monoclonal anti-AdoHcy antibody was added to each well and the samples were incubated at room temperature for 30 min. The plate was then washed 4 times with 350  $\mu$ L of phosphate buffer solution and 100  $\mu$ L of a rabbit anti-mouse-antibody enzyme conjugate was then added to each sample. The plate was next incubated at room temperature for 20 min and each well was washed 4 times with 350  $\mu$ L of phosphate buffer solution. After the addition of 100  $\mu$ L of substrate solution to each sample well, the plate was incubated at room temperature for 10 min. The reaction was stopped by adding 100  $\mu$ L of 0.8 mol/L sulfuric acid and the results were determined within 15 min by the absorbance at 450 nm. The absorbance values inversely correlate with the concentration of AdoHcy and this method showed a reliable recovery rate (90.8–107.4%).

**Dnmt activity.** Mouse aortas were prepared from which nuclear extracts were made using a Nuclear Protein Extraction kit (Beyotime Biotechnology). The protein concentrations were determined using the Bicinchoninic Acid assay kit (Beyotime Biotechnology). We quantified the total Dnmt activity levels using an innovative colorimetric assay with the EpiQuik DNA Methyltransferase Activity Assay kit (Epigentek Group). In this assay, the cytosine-rich DNA substrate is stably coated on the strip wells. These wells are then specifically treated to confer a high DNA adsorption ability. Dnmt transfers a methyl group from S-adenosylmethionine (AdoMet) to a cytosine in the DNA substrate. Methylated DNA can be recognized using an anti-5-methylcytosine antibody. The levels of methylated DNA, which were proportional to the enzymatic activity, were then colorimetrically quantified using an ELISA-like reaction. The results were expressed as absorbance units 450 nm $\cdot$ h $^{-1}$ ·mg protein $^{-1}$ .

**Global DNA methylation status.** DNA was isolated from the aortas of the mice using the Dneasy Tissue kit (Qiagen). After adjusting the sample DNA concentration to 40 mg/L, the purified DNA was immobilized to a strip well with a high affinity for DNA. Assessment of the global DNA methylation status was accomplished using the Methylamp Global DNA Methylation Quantification kit (Epigentek Group). The methylated fraction of DNA was identified using 5-methylcytosine monoclonal antibodies and quantified by an ELISA-like reaction. The levels of methylated

DNA were then proportional to the OD intensity on a microplate reader at 450 nm.

**Statistical analysis.** The results are expressed as the means  $\pm$  SD for each group of mice. Differences among groups were analyzed by 1-way ANOVA with a Tukey's honestly significant difference multiple comparison test when the variances were equal. If the variances were not equal, a rank transformation was carried out prior to ANOVA. Correlations between select pairs of variables were evaluated with the Pearson correlation coefficient and linear regression analysis. A  $P$ -value  $< 0.05$  was considered significant.

## Results

**Body weight and serum B vitamin levels.** After receiving 1 of the 5 experimental diets for 8 wk, the mean body weights of the mice decreased in the dietary groups M-V ( $18.9 \pm 4.1$  g) and C-V ( $20.4 \pm 3.7$  g) compared with group C ( $24.9 \pm 4.0$  g) ( $P < 0.05$ ). The mice fed the M and M+V diets had a similar weight gain to the mice in group C. Dietary intakes did not differ among these groups. In addition, compared with the C diet, a dietary B vitamins deficiency (M-V and C-V diets) or enrichment (M+V diet) caused a significant decrease or increase, respectively, in the serum folate, vitamin B-6, and vitamin B-12 concentrations after 8 wk of feeding (Table 2).

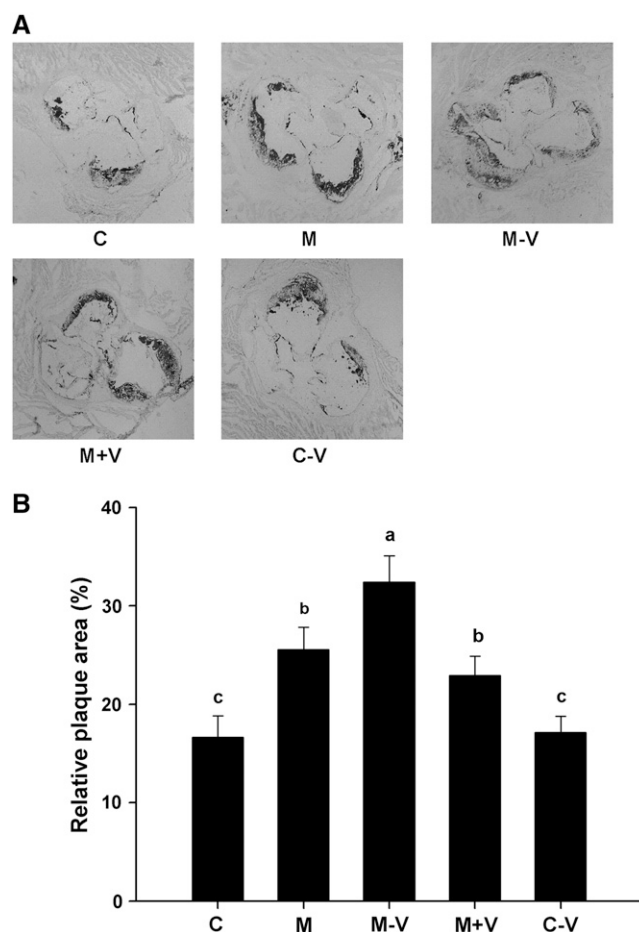
**Atherosclerotic lesions.** We found that the methionine-rich diets increased the aortic sinus plaque areas compared with the control diet. The atherosclerotic lesion areas in the mice from dietary groups M, M-V, and M+V were 53, 95, and 37% greater, respectively, than those in group C ( $P < 0.01$ ) (Fig. 1). Compared with mice in group C, atherosclerotic lesion areas in mice fed the C-V diet did not differ ( $P > 0.05$ ).

**Plasma tHcy and AdoHcy.** Plasma tHcy concentrations were elevated in mice fed the M and C-V diets ( $P < 0.05$ ) and a remarkable increase was observed in the M-V diet mice compared with group C ( $P < 0.001$ ) (Fig. 2A). Two groups (M and M-V) had elevated plasma tHcy concentrations that accompanied the increased plaque sizes. However, mice fed a C-V diet had increased plasma tHcy concentrations but no increase in their lesion areas. In contrast, the plasma tHcy concentrations did not increase in the M+V diet mice, whereas the plaque sizes in this group were larger than those in group C. The concentrations of plasma AdoHcy were elevated as a result of the M, M+V, and M-V diets ( $P < 0.05$ ) (Fig. 2B). Interestingly, the variation in the plasma AdoHcy levels did not parallel that of the plasma tHcy concentrations in our current animal model system. Compared with group C, there was no detectable increment in the plasma AdoHcy concentrations ( $P > 0.05$ ) but a moderate

**TABLE 2** Serum folate, vitamin B-6, and vitamin B-12 concentrations in apoE-deficient mice fed diets varying in methionine and B vitamins for 8 wk<sup>1</sup>

Group	Folate	Vitamin B-6	Vitamin B-12
	nmol/L	nmol/L	pmol/L
C	213.2 $\pm$ 22.0 <sup>b</sup>	123.4 $\pm$ 22.4 <sup>b</sup>	7045 $\pm$ 1618 <sup>b</sup>
M	166.0 $\pm$ 23.1 <sup>c</sup>	87.8 $\pm$ 11.0 <sup>b</sup>	5081 $\pm$ 1136 <sup>bc</sup>
M-V	88.1 $\pm$ 21.4 <sup>d</sup>	42.9 $\pm$ 11.9 <sup>c</sup>	2446 $\pm$ 900 <sup>c</sup>
M+V	293.1 $\pm$ 27.7 <sup>a</sup>	318.8 $\pm$ 62.7 <sup>a</sup>	22082 $\pm$ 4927 <sup>a</sup>
C-V	106.8 $\pm$ 22.8 <sup>d</sup>	20.7 $\pm$ 7.8 <sup>c</sup>	1876 $\pm$ 1042 <sup>c</sup>

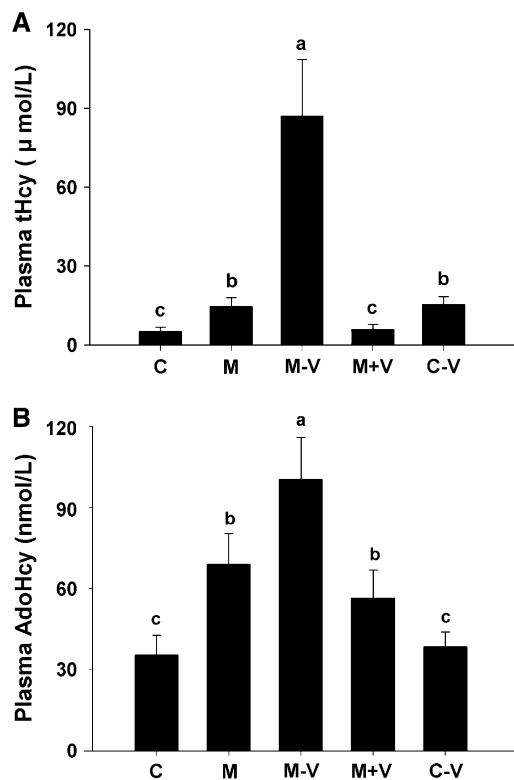
<sup>1</sup> Values are means  $\pm$  SD,  $n = 8$ . Means in a column without a common letter differ,  $P < 0.05$ .



**FIGURE 1** Atherosclerotic lesions on the aortic sinus in apoE-deficient mice fed diets varying in methionine and B vitamins for 8 wk. (A) Cryostat sections of aortic sinuses stained for lipids by Oil red O. Magnification, 10 $\times$ . (B) Relative atherosclerotic plaque areas expressed as the percentage of the total cross-sectional vessel wall area. Values are means  $\pm$  SD,  $n = 10$ . Means without a common letter differ,  $P < 0.05$ .

elevation in the plasma tHcy levels in group C-V. Mice fed the M+V diet had increased plasma AdoHcy concentrations ( $P < 0.05$ ) but normal plasma tHcy concentrations. In addition, the M-V diet caused a severe increase ( $\sim 17$ -fold) in the plasma tHcy concentrations compared with group C ( $P < 0.001$ ) but only a moderate increase in the plasma AdoHcy concentration (2.83-fold) ( $P < 0.001$ ). There was a positive correlation between the plasma AdoHcy concentrations, but not the plasma tHcy levels, and the aortic sinus lesion areas in all of the dietary groups ( $r = 0.866$ ;  $P < 0.001$ ).

**Total Dnmt activity and global DNA methylation analysis.** Compared with the C diet mice, the M-V diet-fed mice had a 28% reduction in Dnmt activity in their aortic tissue ( $P < 0.05$ ) and this was associated with the highest concentrations of plasma AdoHcy (Fig. 3A). In contrast, the C-V diet group had normal AdoHcy concentrations and Dnmt activity did not differ from the C diet-fed mice ( $P > 0.05$ ). We also found a negative correlation between the plasma AdoHcy concentration and Dnmt activity in all groups ( $r = -0.824$ ;  $P < 0.001$ ) but no such correlation between tHcy and Dnmt. Consistent with their decreased Dnmt activity levels, the global DNA methylation status of the aortic tissue was reduced in groups M, M-V, and M+V compared with group C ( $P < 0.01$ ) (Fig. 3B).



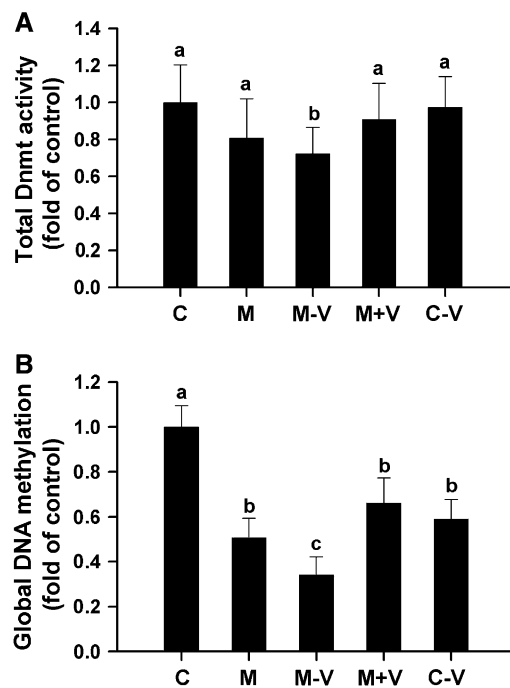
**FIGURE 2** Plasma tHcy (A) and AdoHcy (B) concentration determinations in apoE-deficient mice fed diets varying in methionine and B vitamins for 8 wk. Values are means  $\pm$  SD,  $n = 10$ . Means without a common letter differ,  $P < 0.05$ .

Furthermore, the plasma AdoHcy concentrations were negatively correlated with global DNA hypomethylation in all groups except C-V ( $r = -0.792$ ;  $P < 0.001$ ).

## Discussion

Numerous epidemiological investigations have now demonstrated that a mild elevation in the plasma Hcy concentration is associated with an increased risk of vascular disease and that this is independent of other risk factors (15–21). However, data from other case-control studies do not support such a causal relationship and the mechanism of Hcy-related atherosclerosis still remains insufficiently understood. In fact, it is still unknown whether Hcy is itself a causal factor or just an indirect metabolic marker of some of the biochemical processes that occur in atherosclerosis (22–24). There are a number of intermediates of methionine metabolism, including AdoMet, AdoHcy, and Hcy, and recent studies have shown that a high AdoHcy concentration or low AdoMet:AdoHcy ratio may be more directly related to vascular damage than Hcy (8–10,25). Another study by Troen et al. (7) has reported that the extent of atherosclerosis in apoE-deficient mice is in fact independent of the plasma tHcy concentration. However, the authors in this case did not explore whether the plasma AdoHcy levels correlated with atherosclerotic plaque formation.

In this study, we confirmed that elevated plasma tHcy is not closely associated with the extent of atherosclerotic lesions in our apoE-deficient mice models but that AdoHcy is a good marker of this disease. When these mice were fed the C-V diet, they had no increase in their atherosclerotic lesion areas and a normal range of plasma AdoHcy concentrations but their



**FIGURE 3** Dnmt activity (A) and global DNA methylation status (B) in the aortas of apoE-deficient mice fed diets varying in methionine and B vitamins for 8 wk. Values are means  $\pm$  SD,  $n = 10$ . Means without a common letter differ,  $P < 0.05$ .

plasma tHcy concentrations were moderately elevated. In contrast, atherosclerosis was accelerated in mice fed the M+V diet and they also had elevated plasma AdoHcy concentrations, even though they had normal concentrations of plasma tHcy. This result provided further evidence that the plasma tHcy concentration is not closely associated with atherosclerotic lesions in apoE-deficient mice, consistent with the previous findings of Troen et al. (7). Hence, our data do not support Hcy as an independent risk factor of atherosclerosis. We then analyzed the association of AdoHcy and tHcy with spontaneously atheromatous lesion progression in apoE-deficient mice fed experimental diets that varied in methionine and B vitamin contents. Importantly, the plasma AdoHcy concentrations, but not the plasma tHcy levels, were consistently correlated with the extent of atherosclerosis. Our results thus suggest for the first time, to our knowledge, that plasma AdoHcy is a far better biomarker of atherosclerosis than Hcy and may be causally linked to the pathogenesis of this vascular disease.

Epigenetic changes have been recognized as an important mechanism during oncogenesis, but insufficient data has so far been provided that link this mechanism to the pathophysiology of atherosclerosis (26–28). It is well known that methylation is an important epigenetic feature of DNA that is catalyzed by Dnmt with AdoMet serving as a methyl donor. AdoHcy is a known potent inhibitor of the methyltransferases, including Dnmt, by binding to these enzymes with higher affinity than AdoMet. The accumulation of AdoHcy causes a feedback inhibition of AdoMet-dependent methyltransferases, which can affect the DNA methylation pattern and lead to promotion of chronic diseases (28–31). Recently, Castro et al. (9) confirmed that genomic DNA hypomethylation was strictly dependent on the intracellular AdoHcy content in human umbilical vein endothelial cells. Yi et al. (10) have also demonstrated that increased plasma tHcy content correlates with increased plasma AdoHcy, which in turn



correlates with its intracellular concentration and with the extent of lymphocyte DNA hypomethylation. These findings suggest that the AdoHcy levels are a major predictor of reduced methylation capacity and may be involved in the pathogenesis of chronic diseases.

The results of our study indicate that negative correlations exist between the plasma AdoHcy concentration and Dnmt activity and between the plasma AdoHcy concentration and global DNA hypomethylation in atherosclerotic mice. This in turn indicates that variations in the plasma AdoHcy levels may reflect changes in the intracellular concentrations of this molecule and consequently lead to global DNA hypomethylation in the aortic tissue of this mouse model. Thus, the inhibitory effects of AdoHcy on the methyltransferases may potentially be an important pathogenic mechanism in the development of atherosclerosis. The future determination of intracellular AdoHcy concentrations of aortic tissue will provide direct evidence to support this possibility.

The CpG island hypermethylation of specific genes has been linked to tumor suppressor gene silencing, whereas DNA hypomethylation has been proposed to cause oncogenic mutations by promoting chromosomal instability. The study of epigenetic mechanisms in atherosclerosis has only just begun, and there are few reports that have focused on alterations in the DNA methylation of specific genes related to atherosclerosis. Thus far, the CpG island hypermethylation of the estrogen receptor gene, an antiproliferative gene, has been reported to be associated with atherosclerosis (32). In this study, we did not determine the DNA methylation pattern for specific atherosclerosis-susceptible genes or atherosclerosis-protective genes related to atherosclerotic progression. Subsequent studies will therefore be needed to further validate our findings.

In summary, our study demonstrates that an elevated plasma AdoHcy concentration is a better biomarker of atherosclerosis than the plasma Hcy levels and that AdoHcy may play a role in promoting atherosclerotic lesions in apoE-deficient mice fed a high methionine diet. The mechanisms underlying the atherogenic effects of AdoHcy may also be associated with its inhibition of DNA methylation in aortic tissue.

## Literature Cited

- McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol.* 1969;56:111–28.
- Clarke R. Homocysteine and cardiovascular disease. Overview. *J Cardiovasc Risk.* 1998;5:213–5.
- Scott CH, Sutton MS. Homocysteine: evidence for a causal relationship with cardiovascular disease. *Cardiol Rev.* 1999;7:101–7.
- Boers GH. Mild hyperhomocysteinemia is an independent risk factor of arterial vascular disease. *Semin Thromb Hemost.* 2000;26:291–5.
- Castro R, Rivera I, Blom HJ, Jakobs C, Tavares de Almeida I. Homocysteine metabolism, hyperhomocysteinemia and vascular disease: an overview. *J Inher Metab Dis.* 2006;29:3–20.
- Clarke R, Armitage J. Vitamin supplements and cardiovascular risk: review of the randomized trials of homocysteine-lowering vitamin supplements. *Semin Thromb Hemost.* 2000;26:341–8.
- Troen AM, Lutgens E, Smith DE, Rosenberg IH, Selhub J. The atherogenic effect of excess methionine intake. *Proc Natl Acad Sci USA.* 2003;100:15089–94.
- Kerins DM, Koury MJ, Capdevila A, Rana S, Wagner C. Plasma S-adenosylhomocysteine is a more sensitive indicator of cardiovascular disease than plasma homocysteine. *Am J Clin Nutr.* 2001;74:723–9.
- Castro R, Rivera I, Struys EA, Jansen EE, Ravasco P, Camilo ME, Blom HJ, Jakobs C, Tavares de Almeida I. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. *Clin Chem.* 2003;49:1292–6.
- Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem.* 2000;275:29318–23.
- Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr.* 1993;123:1939–51.
- Xia M, Ling WH, Ma J, Kitts DD, Zawistowski J. Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein e deficient mice. *J Nutr.* 2003;133:744–51.
- Bisp MR, Bor MV, Heinsvig EM, Kall MA, Nexø E. Determination of vitamin B6 vitamers and pyridoxic acid in plasma: development and evaluation of a high-performance liquid chromatographic assay. *Anal Biochem.* 2002;305:82–9.
- Frantzen F, Faaren AL, Alfheim I, Nordhei AK. Enzyme conversion immunoassay for determining total homocysteine in plasma or serum. *Clin Chem.* 1998;44:311–6.
- Ambrosi P, Rolland PH, Bodard H, Barlatier A, Charpiot P, Guisgand G, Friggi A, Ghiringhelli O, Habib G, et al. Effects of folate supplementation in hyperhomocysteinemic pigs. *J Am Coll Cardiol.* 1999;34:274–9.
- Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ Jr, Kohl B, Rao V, et al. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest.* 2001;107:675–83.
- Zhou J, Moller J, Danielsen CC, Bentzon J, Ravn HB, Austin RC, Falk E. Dietary supplementation with methionine and homocysteine promotes early atherosclerosis but not plaque rupture in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol.* 2001;21:1470–6.
- Fujimoto S, Togane Y, Matsuzaki C, Yamashina S, Nakano H, Yamazaki J, Yoshino G. Effects of long-term administration of methionine on vascular endothelium in rabbits. *Nutr Metab Cardiovasc Dis.* 2003;13:20–7.
- Matthias D, Becker CH, Riezler R, Kindling PH. Homocysteine induced arteriosclerosis-like alterations of the aorta in normotensive and hypertensive rats following application of high doses of methionine. *Atherosclerosis.* 1996;122:201–16.
- Zhang R, Ma J, Xia M, Zhu H, Ling W. Mild hyperhomocysteinemia induced by feeding rats diets rich in methionine or deficient in folate promotes early atherosclerotic inflammatory processes. *J Nutr.* 2004;134:825–30.
- Zulli A, Hare DL, Buxton BF, Black MJ. High dietary methionine plus cholesterol exacerbates atherosclerosis formation in the left main coronary artery of rabbits. *Atherosclerosis.* 2004;176:83–9.
- Brattstrom L, Wilcken DE. Homocysteine and cardiovascular disease: cause or effect? *Am J Clin Nutr.* 2000;72:315–23.
- Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. *J Thromb Haemost.* 2005;3:1646–54.
- Ueland PM, Refsum H, Beresford SA, Vollset SE. The controversy over homocysteine and cardiovascular risk. *Am J Clin Nutr.* 2000;72:324–32.
- Loehrer FM, Tschopl M, Angst CP, Litynski P, Jager K, Fowler B, Haefeli WE. Disturbed ratio of erythrocyte and plasma S-adenosylmethionine/S-adenosylhomocysteine in peripheral arterial occlusive disease. *Atherosclerosis.* 2001;154:147–54.
- Dong C, Yoon W, Goldschmidt-Clermont PJ. DNA methylation and atherosclerosis. *J Nutr.* 2002;132:S2406–9.
- Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *J Pathol.* 2002;196:1–7.
- James SJ, Melnyk S, Pogribna M, Pogribny IP, Caudill MA. Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology. *J Nutr.* 2002;132:S2361–6.
- Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet.* 2000;9:2395–402.
- Lee ME, Wang H. Homocysteine and hypomethylation. A novel link to vascular disease. *Trends Cardiovasc Med.* 1999;9:49–54.
- Perna AF, Ingrosso D, Lombardi C, Acanfora F, Satta E, Cesare CM, Violetti E, Romano MM, De Santo NG. Possible mechanisms of homocysteine toxicity. *Kidney Int Suppl.* 2003;S137–40.
- Post WS, Goldschmidt-Clermont PJ, Wilhide CC, Heldman AW, Sussman MS, Ouyang P, Milliken EE, Issa JP. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res.* 1999;43:985–91.