Plasma Homocysteine and Malondialdehyde Are Correlated in an Age- and Gender-Specific Manner

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Homocysteine is an independent risk factor for peripheral vascular and coronary artery disease. The exact mechanism by which homocysteine promotes vascular dysfunction is unclear, but it is speculated to involve oxidative stress. Several studies have investigated the role of homocysteine in promoting oxidative stress and have obtained conflicting results. The age and gender of the subject populations in these studies may have influenced the outcome. Therefore, we investigated whether plasma homocysteine concentrations were correlated with plasma malondialdehyde (MDA, a marker of oxidative stress), and if the subject's age and gender affected this correlation. Plasma homocysteine and MDA were measured in 35 premenopausal women, 14 young men, 38 postmenopausal women, and 18 older men. Homocysteine was significantly higher in men than women ($P < .01$) and in older subjects versus younger. However, MDA was significantly greater only in the young men ($P < .01$). Furthermore, there was a significant correlation between homocysteine and MDA only in these young men ($R^2 = 0.50$, $P < .01$). Lastly, subjects undergoing a methionine load did not exhibit increased MDA despite significant increases in homocysteine. Since oxidative stress correlates with basal homocysteine only in young men and does not increase with acutely increased homocysteine, it is unlikely to be the result of a direct effect of homocysteine.

ELEVATED PLASMA homocysteine is an independent risk factor for peripheral vascular and coronary artery disease.[1-3] However, this risk has been reported to be strongest among young men with few or no other noteworthy risk factors.[4,5] Plasma homocysteine concentrations normally increase with age and are increased in men compared to women.[1,6] However, after menopause this gender difference in plasma homocysteine concentration is decreased or negated. Normally, premenopausal women will have 20% lower homocysteine concentrations than men of the same age.[1,7] After menopause, homocysteine concentrations increase and approach or are equal to that of men of similar age.[7] These specific fluctuations in homocysteine concentration have been proposed to be estrogen-related and may partially explain estrogen's protective effect on the cardiovascular system in premenopausal women.[8]

One mechanism by which increased homocysteine has been proposed to influence cardiovascular risk is by promoting increased oxidative stress.[9-11] However, there is equal evidence for and against the homocysteine-oxidative stress hypothesis. Early in vitro studies using bovine aortic endothelial cells demonstrated that increased homocysteine in the presence of copper could increase cell damage via the formation of hydrogen peroxide.[12] However, other studies have failed to observe evidence of increased oxidative stress in vivo among patients with increased plasma homocysteine compared to controls.[13-15] Nevertheless, there is evidence of increased in vivo lipid peroxidation in certain animal models of hyperhomocysteinemia (nitrous oxide–treated pigs and methionine-fed rabbits), as well as methionine-loaded human volunteers.[10,16-18] Therefore, the issue of whether homocysteine promotes increased oxidative stress in vivo, and thereby increases the risk of cardiovascular disease, is unresolved.

The focus of this study was to investigate whether homocysteine is correlated with a marker of in vivo oxidative stress (malondialdehyde [MDA]), and whether the gender and age of the subjects studied would influence this correlation.

MATERIALS AND METHODS

Subjects

One hundred five subjects (14 young men, 35 premenopausal women, 18 older men, and 38 postmenopausal women) were recruited for this study. The study was approved by the hospital institutional review board and all subjects gave informed consent. The postmenopausal women were without menstrual bleeding for at least 12 months prior to the study (average, 10 years; range, 1 to 34 years) and had not used hormone-replacement therapy for at least 3 months prior to study entry. Blood samples from the premenopausal women were collected during the second week after the start of their last menstrual period to standardize the effect of steroid hormones. All subjects were healthy without chronic hypertension, renal or metabolic disease. The subject's self-reported cigarette use was as follows: 4 of 35 younger women, 3 of 14 younger men, 4 of 38 older women, and 4 of 18 older men.

Blood Samples

All plasma samples were handled similarly. Samples were obtained after an overnight fast. Plasma was immediately prepared from blood anticoagulated with EDTA. Sample aliquots were prepared under sterile conditions and stored at −80°C until assayed.

Homocysteine Determination

Total plasma homocysteine was analyzed according to the procedure of Jacobsen et al.[19] In brief, samples (100 μL) were mixed with 10 μL of water and 5 μL of n-amyl alcohol. Subsequently, 35 μL of 1.43-mol/L $NaBH_4$ in 0.1 mol/L NaOH was added to reduce disulfide bonds and, after vortexing, 35 μL of 1N HCl added. After mixing, the thiols were derivatized by incubating at 42°C for 12 minutes with 50 μL of 10-mmol/L monobromobimane (thiolyte; Calbiochem) in 4 mmol/L NaEDTA, pH 7. The samples were cooled to room temperature and then mixed with 50 μL of 1.5-mol/L perchloric acid. The samples were left at room temperature for 10 minutes and then microcentrifuged for

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Table 1. Baseline and Clinical Measures of the Subject Groups

	Premenopausal Women (n = 35)	Young Men (n = 14)	Postmenopausal Women (n = 38)	Older Men (n = 18)
Age (yr)	24.9 ± 6.8†‡	28.7 ± 6.1†‡	57.2 ± 3.1	59.2 ± 3.6
BMI (kg/m^2)	22.5 ± 3.2†‡	25.4 ± 6.4	26.8 ± 4.8	27.1 ± 2.7
Homocysteine (μmol/L)	8.4 ± 1.6*‡	10.6 ± 1.9	9.6 ± 2.1‡	12.4 ± 3.1
MDA (μmol/L)	0.47 ± 0.14*	0.61 ± 0.24†	0.46 ± 0.11	0.49 ± 0.12
Triglycerides (mg/dL)	97.1 ± 43.3	144.8 ± 85.2	132.7 ± 67.3	142.8 ± 83.7
Total cholesterol (mg/dL)	172.2 ± 41.7†‡	171.2 ± 24.6†‡	238.5 ± 42.2	228.0 ± 37.7
LDL cholesterol (mg/dL)	96.9 ± 34.1†‡	93.0 ± 19.7†‡	149.1 ± 37.5	148.3 ± 31.8
HDL cholesterol (mg/dL)	56.2 ± 9.5	49.2 ± 11.7†	62.9 ± 14.8‡	51.0 ± 12.5

NOTE. Data are means ± SD. Duplicate determinations were done for each laboratory measure.

*Different from young men, †different from postmenopausal women, and ‡different from older men; for all, $P < .05$ after controlling for multiple comparisons following cell means ANOVA model.

10 minutes. Twenty microliters of 2-mol/L Tris was carefully added to the tubes, which were then gently inverted to avoid disrupting the pellets. The samples were next microfuged for 4 minutes.

Aliquots (20 μL) were separated on a 4.6 × 150 mm (5-μm particle) Prodigy column (Phenomenex, Torrance, CA) maintained at 30°C. Eluant A was water:methanol:acetic acid (94.5:5.0:0.5 by volume). Eluant B was 100% methanol. Total flow rate was 1 mL/min and the solution gradient involved minor modification of the Jacobsen et al[19] method: 0 to 1 minute, 10%B; 1 to 6 minutes, 10-35% B; 6 to 7.3 minutes, 35%B; 7.3 to 8 minutes, 35-100%B; 8 to 9 minutes, 100%B; 9 to 10 minutes, 100-55%B; 10 to 11 minutes, 55-10%B; and 11 to 12 minutes, 10%B. The thiol derivatives were detected fluorimetrically with excitation at 390 nm and emission at 470 nm.

Calibration curves were generated for every assay and were included at the beginning and end of each analytical set. They consisted of normal human plasma spiked with 0, 2.5, 5, 7.5, 10, 15, 20, and 25 μmol/L L-homocystine. The coefficient of variation between assays was 8%.

Methionine Load

The methionine load consisted of an oral dose of L-methionine (fit for human consumption; Ajinomoto Co, Teaneck, NJ; 0.1 g/kg body weight) administered in orange juice. Subjects were then restricted to a low-methionine snack (bagels, an apple or pear, and beverages including juice) for the next 6 hours until after the next blood sample was collected. After the collection of the second blood sample, subjects were allowed to return to their normal diet, but fasted for 12 hours before the third and final blood sample was collected (24 hours after receiving the methionine load).

Assessment of Lipid Peroxidation

MDA, a metabolite of lipid peroxides detectable in plasma, was used as an indicator of lipid peroxidation. Plasma MDA concentrations were determined with the high-pressure liquid chromatography (HPLC) procedure of Wong et al as previously reported.[20] In this assay, the authentic chromogen produced by the reaction of MDA with thiobarbituric acid is separated from other interfering chromogens and quantified. The detection limit of the assay is 0.15 μmol/L. Interassay and intra-assay variations have been 12% and 5%, respectively.

Lipid Measurements

All lipid measurements for total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were performed by a certified nutrition laboratory at the University of Pittsburgh Graduate School of Public Health.

Statistical Methods

Means and standard deviations are reported. Differences in homocysteine, MDA, body mass index (BMI), triglycerides, total, LDL, and HDL cholesterols were analyzed with 2-way analysis of variance (ANOVA) for the effects of gender, age, and their interaction. Age had 2 levels: younger (≤45 years) and older. Specific pairwise comparisons were performed among the groups defined by gender and age using a cell means ANOVA method as described by Stata Corp (College Station, TX). Linear regression was used to control for possible confounding variables. Changes in homocysteine and MDA during the methionine load were analyzed by repeated-measures ANOVA with Bonferroni/Dunn post-hoc testing to identify differences between the sampling times. ANOVA, linear regression, and correlation analysis were done using Stata statistical software and the repeated-measures ANOVA was done using Statview 5.1 software. All data reported are the mean of duplicate determinations for each measure. Statistical significance was accepted at $P < .05$.

RESULTS

The results of the 4 group comparisons using the cell means ANOVA method are presented in Table 1. The mean plasma concentration of total homocysteine differed by age and gender. Homocysteine concentrations increased with age ($P = .02$) and were higher in men ($P = .002$) than in women. Homocysteine was higher in older men compared to older and younger women, and was higher in young men compared to young women (Table 1 and Fig 1). Age and gender remained significantly related to homocysteine after multiple variable linear regression controlling for MDA, triglycerides, total cholesterol, and BMI ($P < .05$). Plasma MDA concentrations were significantly higher in men ($P = .003$), and this effect remained after controlling for BMI. Young men had greater MDA concentrations than the postmenopausal and premenopausal women (Table 1 and Fig 2).

We investigated the possible influence of homocysteine on oxidative stress by testing for correlations between homocysteine and MDA. Overall, homocysteine was not correlated with MDA. However, among the 4 subject groups studied, homocysteine was significantly correlated with MDA only in the group of young men ($R^2 = .50$, $P < .01$) (Fig 3).

Plasma triglycerides, cholesterol, and LDL cholesterol can serve as substrates for lipid peroxidation and higher concentrations of these substrates can increase MDA concentrations.

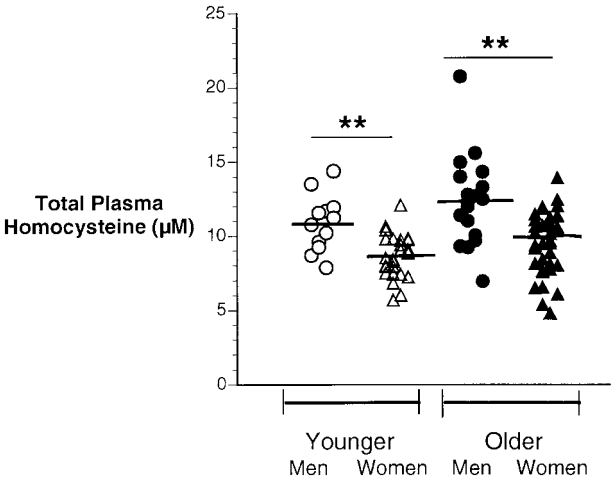

Fig 1. Total plasma homocysteine concentrations in young men, premenopausal women, postmenopausal women, and older men. Individual points represent the mean of duplicate determinations. Mean value indicated by the solid bar. **$P < .001$.

Therefore, we measured plasma triglycerides, and total and LDL cholesterol. Triglyceride concentrations increased with age ($P = .04$) and were higher in men ($P = .04$). Total cholesterol and LDL cholesterol increased with age ($P = .001$, $P < .001$, and $P = .04$, respectively) but were not different by gender (Table 1). However, lipid concentrations did not correlate with MDA. Furthermore, homocysteine was not correlated with any of the lipid measures. Thus, it does not appear that the observed correlation between homocysteine and MDA among the younger men is explained by differences in plasma lipids.

To further explore the association between homocysteine and MDA, a subset of the young men and premenopausal women participated in a methionine-loading test. As shown in Table 2, the methionine load induced a significant increase in plasma homocysteine by 6 hours after the administration of methionine. Furthermore, although homocysteine concentrations had fallen by 24 hours after the load, homocysteine concentrations were still twice that of the first fasted sample. Despite the significant increase in homocysteine over 24 hours, there was no change in plasma MDA (Table 2).

DISCUSSION

The focus of this study was to investigate whether homocysteine was correlated with an in vivo marker of oxidative stress (MDA) among a cohort of subjects taking into account the subject's age and gender. Our data confirm prior reports that homocysteine is different between men and women, and that plasma homocysteine normally increases with age.[1,7] When individual groups were compared, homocysteine concentrations in men were significantly higher than in age-matched premenopausal women. Given the number of subjects in our study, power analysis indicates that we should be able to determine a mean difference in homocysteine of 2.4 μmol/L with 90% power with an alpha of 0.01. The difference in homocysteine concentration between men and women was decreased or lost after menopause.[7,8] The mechanism responsible for the noted differences in homocysteine between men and women and older and younger subjects is likely to involve several consequences of aging, since homocysteine increases with age in men and women.[21,22] In women especially, these likely include hormone-specific effects related to menopause, since estrogen has been shown to affect plasma homocysteine.[23,24] Previous studies investigating the association between homocysteine and oxidative stress in human subjects have focused on men. However, based on changes in plasma homocysteine concentrations between men and women as they age, we postulated that the age and/or gender of the subject population being investigated might influence the results of these studies.

Contrary to our expectations, MDA concentrations were highest among the subset of young men with all other groups not different. This result is somewhat puzzling since oxidative stress and MDA have previously been reported to increase with age.[25-28] MDA has been used extensively as a measure of oxidative stress, and the HPLC method is reported to be a very sensitive method of detection since it eliminates other possible contaminating chromogens.[20,27] On the other hand, it is possible that MDA may be derived from other sources other than lipid peroxidation such as a byproduct of thromboxane synthesis during platelet activation.[29,30] However, platelet activation is not reported to be higher in young men compared to other normal subject groups.[31] Another possibility might be that the group of young men are more physically active than the other groups, and this increased activity may result in increased markers of oxidative stress as is reported to occur in exercise studies.[32-34] In the absence of testing these alternative explanations, we lack definitive explanation for this result.

In our analysis of the correlation between homocysteine and MDA, we observed a strong association between homocysteine and MDA only in the young men. Power analysis indicates that

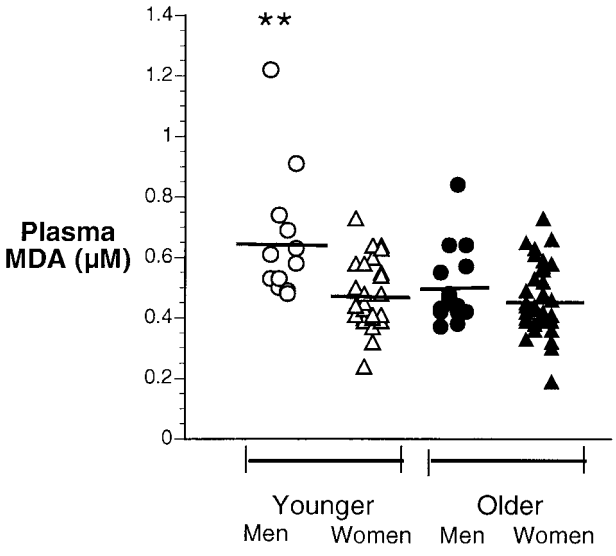

Fig 2. Plasma MDA concentrations in young men, premenopausal women, postmenopausal women, and older men. Individual points represent the mean of duplicate determinations. Mean value indicated by the solid bar. **$P = .02$.

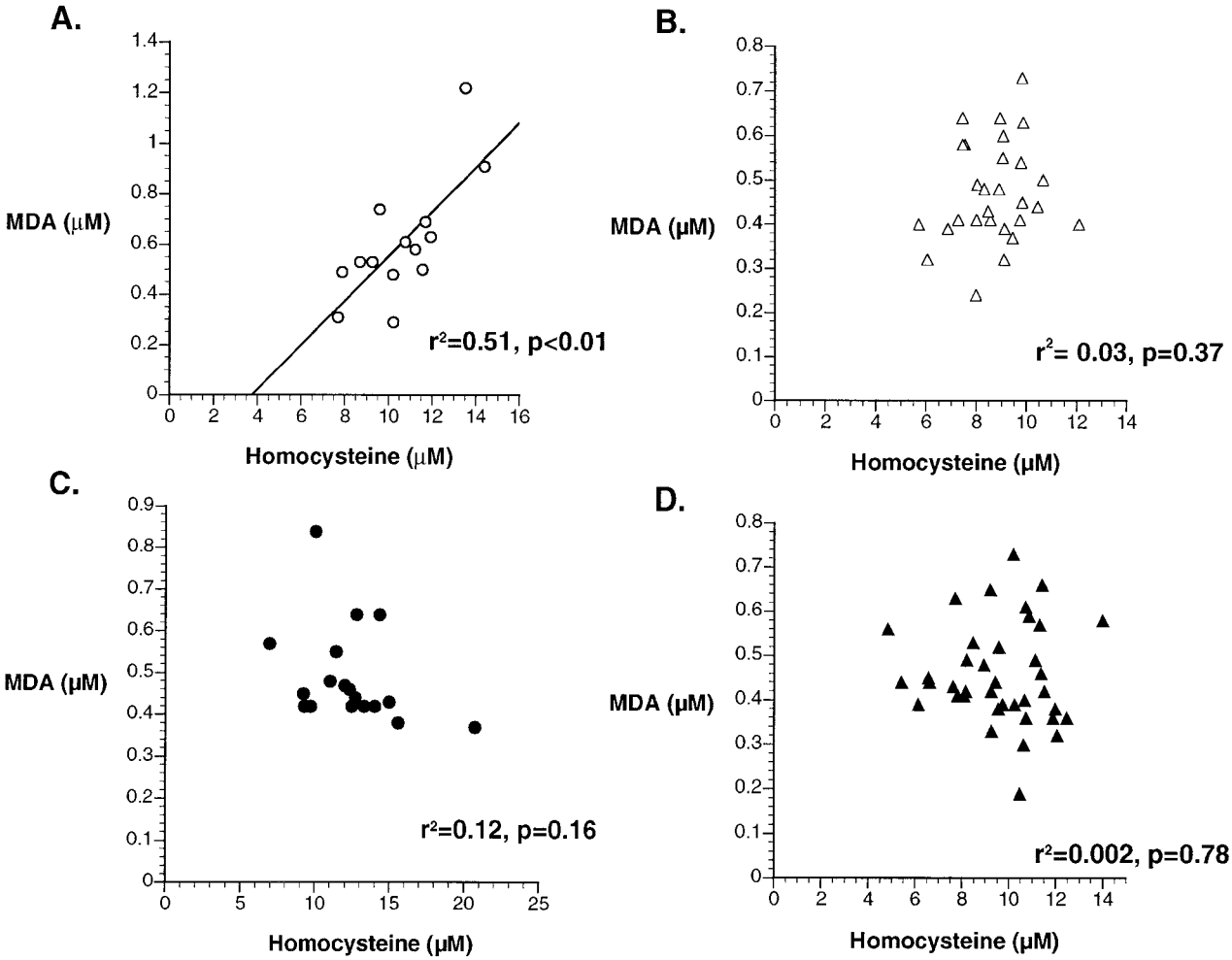

Fig 3. Correlations between homocysteine and MDA in (A) young men (note that after removal of the greatest MDA value a significant correlation persists, $R^2 = 0.46$, $P < .01$), (B) premenopausal women, (C) older men, (D) postmenopausal women.

with the number of subjects in the other groups we could have detected (alpha of 0.05 and 80% power) even a fairly weak correlation at an R^2 value of 0.2. This result is consistent with a previous study that also reported a significant association between homocysteine and in vivo markers of oxidative stress among men.[35] These data are also reminiscent of the results of some prospective studies investigating the risk of cardiovascular disease among subjects with pre-existing elevations in homocysteine,[4,5] which indicated that elevated homocysteine imparts the highest risk of future cardiovascular disease among young men with few or no other risk factors. However, since this correlation was observed in a small subset of subjects, we further tested it by directly altering subject's homocysteine concentrations and assaying for additional changes in oxidative stress.

We used methionine loading to acutely increase homocys-

Table 2. Transient Increases in Homocysteine Do Not Lead to Increases in MDA

	Fasting	6 Hours Post	24 Hours Post
Homocysteine (μmol/L)			
Young men (n = 12)	10.9 ± 1.9	35.1 ± 7.9*	20.0 ± 6.8*
Premenopausal women (n = 28)	8.7 ± 1.4	31.1 ± 9.4*	14.2 ± 3.6*
MDA (μmol/L)			
Young men (n = 12)	0.66 ± 0.22	0.54 ± 0.16	0.64 ± 0.13
Premenopausal women (n = 28)	0.49 ± 0.11	0.50 ± 0.19	0.51 ± 0.11

NOTE. Data are means ± SD. Duplicate determinations were done for each laboratory measure.
*$P < .0001$ compared to paired fasting samples.

teine concentrations to test whether increased homocysteine in young men and women would be associated with parallel increases in MDA as reported previously.[36] Despite significant increases in homocysteine, MDA concentrations remained unchanged over a 24-hour period. These results conflict with those of Domagala et al who report finding significant increases in MDA after a methionine load.[36] Similar to our study, Domagala et al investigated the effect of methionine loading on both men and women. However, it is possible that the differences between these studies relate to different measures of oxidative stress. Domagala et al evaluated oxidative stress by measuring changes in all thiobarbituric acid–reactive substances (TBARS), which identifies many substances other than MDA,[20] whereas we have evaluated changes in oxidative stress using an HPLC method that separates MDA from other interfering chromogens.[20] In addition, our results agree well with those of Nightingale et al,[31] who reported finding no association between homocysteine and several markers of oxidative stress in a group of men who received either a placebo or a methionine load. A study by Kanani et al suggested that acute alterations in vascular response with increased homocysteine may be secondary to oxidative stress.[10] In this study endothelial-mediated relaxation was reduced after a methionine load and restored by ascorbate administration.[10] However, markers of oxidative stress were not measured. Since ascorbate may affect vascular responses by mechanisms other than preventing oxidative stress and since we did not measure vascular responses, the question remains open. Nonetheless the weight of evidence favors no acute increase in oxidative stress with methionine loading. Therefore, we conclude that although homocysteine may be correlated with in vivo markers of oxidative stress, the age and gender of the subject population affect this correlation. Furthermore, the strong correlation between homocysteine and MDA among young men is either not causal or at least not a rapid and direct effect since transient increases in homocysteine in the same subjects failed to alter MDA concentrations. We propose that this correlation likely points to a common intermediate between these 2 molecules and that homocysteine is unlikely to be a significant mediator of oxidative stress in vivo.

The question of whether elevated homocysteine promotes oxidative stress and by this mechanism may contribute to the greater risk of future cardiovascular disease has been debated for years with abundant evidence presented by both sides. Supporters of this hypothesis point to the ability of thiols such as homocysteine to auto-oxidize with consequent free radical generation, the presence of markers of oxidative stress in animal models of hyperhomocysteinemia, the ability of increased anti-oxidants such as ascorbate to eliminate the effect of elevated homocysteine on vascular function in vivo, and the association between homocysteine and F_2-isoprostane concentrations (a marker of oxidative stress).[10,35,37-39] Conversely, opponents of this hypothesis point out the persistent lack of evidence of increased oxidative stress among patients with severe hyperhomocysteinemia, that homocysteine is normally a very small percentage of the total plasma thiol pool and is therefore likely a minor source of thiol-mediated oxidant stress, and the lack of an increase in markers of oxidative stress after a methionine load despite significant increases in homocysteine.[13-15,31] This debate was recently reviewed by Jacobsen,[40] and despite finding a correlation between homocysteine and MDA among young men, the results of this study go further to support the opponents of the homocysteine/oxidative stress hypothesis.

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