Plasma homocysteine levels increase following stress in older but not younger men

Running title: Homocysteine increases following stress in older but not younger men

Ulrike Kuebler[1], Michael Linnebank[2], Alexander Semmler[2], Birgit Stoffel-Wagner[3], Roberto La Marca[1], Ulrike Ehlert[1], & Petra H. Wirtz[4*].

[1]*Department of Clinical Psychology and Psychotherapy, University of Zurich, Zurich, Switzerland.*

[2]*Department of Neurology, University Hospital Zurich, Zurich, Switzerland.*

[3]*Institute of Clinical Chemistry and Clinical Pharmacology, University Bonn, University Hospital of Bonn, Bonn, Germany*

[4]*Biological and Health Psychology, University of Bern, Bern, Switzerland.*

*Address for correspondence:

Petra H. Wirtz, PhD, University of Bern, Department of Psychology, Biological and Health Psychology, Alpeneggstrasse 22, 3012 Bern, Switzerland, Email: petra.wirtz@psy.unibe.ch, Tel: +41 31 631 5790 / Fax: +41 31 631 4155

Abstract

Background: The incidence and prevalence of cardiovascular disease (CVD) increases with age. Some evidence suggests that mental stress may increase plasma homocysteine (Hcy), an amino acid relating to CVD. However, none of these studies assessed age effects on Hcy stress reactivity, nor did they control for age. The objective of this study was a) to investigate whether Hcy reactivity to psychosocial stress differs between younger and middle-aged to older men and b) to study whether psychosocial stress induces Hcy increases independent of age.

Methods: 28 younger (20-30 yrs) and 22 middle-aged to older (47-65 yrs) apparently healthy men underwent an acute standardized psychosocial stress task combining public speaking and mental arithmetic in front of an audience. Blood samples for Hcy measurements were obtained immediately before and after, as well as 10 and 20 minutes after stress. Moreover, salivary cortisol was repeatedly measured to test the effectiveness of the stress task in triggering a neuroendocrine stress response.

Results: Hcy reactivity to stress differed between age groups ($F(1.4, 60.7) = 5.41$, $p = .014$). While the older group displayed an increase in the Hcy response to stress ($F(2.5, 39.8) = 3.86$, $p = .022$), Hcy levels in the younger group did not change ($p = .27$). Psychosocial stress per se did not change Hcy levels independent of age ($p = .53$).

Conclusions: Our findings suggest that psychosocial stress does not evoke an Hcy response per se, but only in interaction with age pointing to a mechanism by which mental stress may increase CVD risk in older individuals.

Key words: homocysteine, age, stress reactivity, acute mental stress, TSST, cardiovascular disease risk

Introduction

The incidence and prevalence of atherosclerotic and thrombotic cardiovascular disease (CVD) increases progressively with age (Herrera et al., 2010). To explain the higher atherothrombotic risk and ultimately cardiovascular mortality with increasing age, several studies have attempted to elucidate changes in atherogenic and thrombogenic factors during aging (Weinsaft and Edelberg, 2001; Torregrossa et al., 2011).

Homocysteine (Hcy) has been firmly established as an independent predictor of atherosclerotic and thrombotic cardiovascular disease (CVD) (McCully, 1996; Wald et al., 2002). Moreover, plasma Hcy tends to rise in healthy persons parallel with age (Ellinson et al., 2004). Hcy is a sulfur-containing amino acid derived from the dietary amino acid methionine (Castro et al., 2006). Hcy plasma levels are regulated by a complex metabolic pathway involving genetics, nutrition, and renal function (Refsum et al., 1998; Castro et al., 2006). Notably, the age-related increase in Hcy levels may result from suboptimal intake or absorption of vitamin B6, B12, and folate acting as key cofactors for Hcy metabolism, and decreased renal clearance of Hcy (Selhub et al., 1993; Castro et al., 2006). The mechanisms by which elevated Hcy levels relate to CVD are not fully understood. However, compelling evidence from experimental models indicates that Hcy is responsible for arterial changes and vascular damage by impairing bioavailability of nitric oxide and antioxidant defense, enhancing lipid peroxidation (Papatheodorou and Weiss, 2007), causing protein modification (Jakubowski, 2001, 2004), and thrombosis (Undas et al., 2006; Jakubowski et al., 2008), or activating inflammation (Jakubowski, 2005; Bogdanski et al., 2008). Notably, abundant epidemiological and experimental evidence suggests a potential causal role of mild to moderate hyperhomocysteinemia in the pathogenesis of CVD (McCully, 1996; Wang et al., 2007; Dragani et al., 2012). However, recent intervention trials have raised suspicion about this notion as treatment of mild hyperhomocysteinemia with B vitamins resulted in lower Hcy

levels without subsequent reduction of CVD incidence (Clarke et al., 2010). Interestingly, the vitamin B induced Hcy reduction was associated with reduction of stroke risk (Saposnik et al., 2009). In addition, some Mendelian randomization studies found an increased CVD risk in subjects with lifelong mildly to moderately elevated plasma Hcy due to polymorphisms in Hcy-metabolizing enzymes (Wald et al., 2002; Casas et al., 2005; Cronin et al., 2005), whereas others did not detect an association (Clarke et al., 2012). Notably, intervention trials as well as Mendelian randomization studies have several limitations (for review see (Smulders and Blom, 2011)) and results need to be interpreted with care.

In addition to age, Hcy, and their interaction, mental stress is a further factor that has been associated with CVD risk. Accumulating evidence indicates a strong impact of mental stress on the pathogenesis of atherosclerotic and thrombotic CVD (Brotman et al., 2007). It has been suggested that repeated episodes of acute or chronic stress may initiate or promote the atherosclerotic process via effects on Hcy (Black and Garbutt, 2002). Indeed, a study assessing basal Hcy levels in non-smoking war veterans with and without Post Traumatic Stress Disorder (PTSD) found highest Hcy levels in war veterans with PTSD (Jendricko et al., 2009). As PTSD is likely to add to chronic stress load, the study's findings point to a potential association between stress and higher Hcy plasma levels. In terms of acute stress reactivity, studies in rodents show increases in plasma Hcy immediately after restrained stress (de Oliveira et al., 2004; de Souza et al., 2006). However, human studies investigating Hcy reactivity to acute stress are rare and results are contradictory. While two studies reported increases in plasma Hcy levels in response to acute mental stress in middle-aged to older women (Stoney, 1999) and in young men (Sawai et al., 2008), another study including pre- und postmenopausal women found no Hcy stress reactivity (Farag et al., 2003). Moreover, none of these studies assessed age effects on Hcy stress reactivity, nor did they control for age. To date, it has not yet been investigated whether a potential Hcy change following acute

mental stress might be age-dependent, which could shed light onto the complex mechanisms in the interface between age, Hcy, stress reactivity, and their interactions.

Therefore, we aimed to elucidate whether a potential Hcy stress reactivity differs between younger and middle-aged to older men. We hypothesized that older men show higher Hcy responsiveness to acute stress than younger men. Moreover, we investigated whether Hcy levels do change following mental stress independent of age.

Methods

Participants

This study is part of a larger project assessing psychobiological stress reactivity in healthy men (Wirtz et al., 2008). The Ethics Committee of the State of Zurich, Switzerland, formally approved the research protocol. For the purpose of this part of the study we intentionally selected from an existing study sample of N = 63 apparently healthy men aged between 20 to 65 yrs a group of younger men without age-related CVD risk (age range 20-30 yrs) and a group of middle-aged to older men with age-related CVD risk (age range 45-65 yrs) for analysis of Hcy levels from frozen plasma samples. Notably, the threshold of 45 yrs of age for increased age-related CVD risk was based on previous literature (Roger et al., 2011). Applying these age criteria for group inclusion the final sample of this study comprised 28 subjects aged 20 to 30 yrs ("younger men") and 22 subjects aged from 47 to 65 yrs ("older men") rendering a total sample size of N=50. All subjects provided written informed consent.

The study was conducted between April 2004 and August 2005. In the main study, we intentionally recruited nonsmoking men aged between 20 and 65 years who were in good physical and mental health as confirmed by a telephone interview using an extensive health questionnaire. Specific exclusion criteria were obtained from the subjects' self-report and

included clinical psychosomatic and psychiatric diseases, regular strenuous exercise, alcohol and illicit drug abuse, any heart disease, varicosis or thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, liver, and renal diseases, chronic obstructive pulmonary diseases, allergies and atopic diathesis, rheumatic diseases, and current infectious diseases. In addition, participants were included only if they reported taking no prescribed and/or over-the-counter medication, either regularly or occasionally. In case of inconclusive personal or medication history, the subjects' primary care physician was contacted for verification.

Study design

Subjects were tested between 1400h and 1600h. The night before the test, they abstained from physical exercise, alcohol, and caffeinated beverages. To inflict acute psychosocial stress, we applied the standard protocol of the widely used Trier Social Stress Test (TSST) combining a five minute preparation phase followed by a five minute mock job interview, and a five minute mental arithmetic task in front of a panel of one man and one woman (Kirschbaum et al., 1993). The TSST has been shown to reliably induce profound endocrine and cardiovascular responses (Kirschbaum et al., 1993; Dickerson and Kemeny, 2004). After test completion, subjects remained seated in a quiet room for 60 minutes.

Blood samples for assessment of Hcy were taken immediately before (baseline) and after, as well as 10, and 20 minutes after completion of the TSST. For vitamin B12, folate, and creatinine, another blood sample was taken at baseline. For determination of salivary free cortisol levels, samples of saliva were collected immediately before (baseline) and after, as well as, 10, 20, 30, 45, and 60 minutes after TSST completion. BP was measured immediately before and 30 minutes after stress by sphygmomanometry (Omron 773, Omron Healthcare

Europe B.V. Hoofddorp, Netherlands) and mean arterial pressure (MAP) was calculated by the formula (2/3×mean diastolic BP)+(1/3 mean systolic BP).

Biochemical analyses

For analyses of Hcy, vitamin B12, folate, and creatinine, venous blood was drawn through an indwelling forearm catheter into EDTA-coated monovettes (Sarstedt, Numbrecht, Germany), and immediately centrifuged for 10 minutes at 2000 x g and 4°C. Obtained plasma was stored at -80°C until analysis.

Hcy was determined by fully automated particle-enhanced immunonephelometry with a BN II System (Siemens Healthcare Diagnostics, Eschborn, Germany) by enzymatic conversion to S-adenosyl-homocysteinee (SAH). Inter- and intra-variance was 5.6% and 3.4%, respectively.

Plasma concentrations of vitamin B12 and folate were measured by means of a competitive chemiluminescent immunoassay with an Access™ Immunoassay System (Beckman Coulter, Krefeld, Germany) according to the manufacturer's instructions. Inter- and intra-variance of the vitamin B12 assay was 4.2% and 3.8%, respectively. Inter- and intra-variance of the folate assay was 3.6% and 3.1%, respectively.

Plasma creatinine was determined on the Dimension VistaTM clinical chemistry system (Siemens Healthcare Diagnostics) with a commercially available assay based on a modification of the Jaffé method according to the manufacturer´s instructions. Inter- and intra-variance was 2.5% and 1.2%, respectively.

For cortisol, saliva samples were collected in Salivettes (Sarstedt, Sevelen, Switzerland) and stored at -20°C until analysis. Centrifugation of thawed saliva samples was at 3000 x g, yielding low-viscosity saliva. Free cortisol concentrations were determined using

a commercial chemiluminescence immunoassay (LIA) with high sensitivity of 0.16 ng/ml (IBL Hamburg, Germany). Inter- and intra-variance was < 11.5% and 7.7%, respectively.

Statistical analyses

Data were analyzed using SPSS Inc. version 17.0 for Windows (Chicago, IL, USA) and presented as mean ± SEM. All tests were two-tailed with the significance level set at $p \leq .05$. G*Power 3.1 analysis revealed that the optimal total sample size to predict group differences in Hcy stress reactivity was $N = 46$ for detecting a small effect size of $f = 0.10$ in repeated measurement analysis of variance (ANOVA; with Hcy as repeated factor with four measurements that intercorrelate >.90) with a power of .95. Prior to statistical analyses, data were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests. Skewed Hcy values were logarithmically transformed to approach normal distribution.

Differences between the characteristics of the two subject groups were calculated using univariate analysis of variance (ANOVA; Table 1).

To test whether the stressor evoked a significant neuroendocrine stress response, we calculated repeated measures ANOVA with group (younger vs. older men) as the independent variable and the eight time points in which cortisol was measured as repeated dependent variable.

In order to investigate age effects on Hcy stress reactivity, we calculated repeated measures ANCOVA with group (younger vs. older men) as independent variable and the four Hcy measurements as repeated dependent variable. Post-hoc testing of significant Hcy effects comprised separate recalculations of repeated ANCOVAs for each group.

To test for overall Hcy stress reactivity independent of group i.e. age, we further performed repeated measures ANCOVA with both groups combined to one single group as independent variable and the four Hcy measurements as repeated dependent variable, while controlling for age as covariate. Because of known associations with age (Roberts and Williamson, 2002; Higashi et al., 2012), we controlled in all ANCOVAs of Hcy data for possible confounding effects of the CVD risk factors body mass index (BMI) and MAP by including these variables as covariates. In light of previously reported associations between vitamin B12, folate, and creatinine (Hcy confounders) with plasma Hcy levels at rest (Refsum et al., 1998; Castro et al., 2006), we additionally controlled for Hcy confounders in Hcy analyses. Moreover, in complementary analyses testing for group differences in Hcy stress reactivity we additionally accounted for baseline differences in Hcy levels by subtracting baseline Hcy levels from all Hcy measurements and repeated the respective ANCOVA analysis.

Effect size parameters (*f*) were calculated from partial η^2-values and are reported where appropriate (effect size conventions: *f*: .10 = small, .25 = medium, .40 = large).

Results

Subject's characteristics

Table 1 provides the characteristics of subjects in the younger and older age group. BMI, MAP and plasma levels of Hcy at rest were higher in older as compared to younger subjects. In addition, the older group had lower baseline levels of salivary cortisol (p = .041). There were no group differences in plasma levels of vitamin B12, folate and creatinine.

Validation check of the stress protocol

ANOVA for repeated measurements revealed significant salivary cortisol increases in response to stress across both age groups (main effect of stress in the total group: $F(2.7/125.9) = 29.39$, $p = <.001$, $\eta^2 = .39$; main effect of stress in the younger group: $F(2.6/67.7) = 11.73$, $p < .001$, $\eta^2 = .31$; main effect of stress in the older group: $F(1.6/32.3) = 26.90$, $p < .001$, $\eta^2 = .57$)) (Fig. 1). Stress reactivity differed significantly between groups (interaction group-by-stress: $F(2.7/125.9) = 4.51$, $p = .006$, $\eta^2 = .09$) with higher stress reactivity in the older group.

Please insert figure 1 around here.

Homocysteine stress reactivity

Repeated measures ANCOVA revealed that Hcy reactivity to stress differed between age groups (interaction age-by-stress: $F(1.3, 60.3) = 3.89$, $p = .042$, $\eta^2 = .08$, $f = 0.29$). This effect became even stronger when controlling for the full set of confounders (interaction age-by-stress: $F(1.4, 60.7) = 5.41$, $p = .014$, $\eta^2 = .11$, $f = 0.35$) (Fig. 2). With exception of BMI ($p = .038$), none of the confounders significantly related to Hcy stress reactivity (p's>.16). Additional adjustment of repeated Hcy measures to Hcy baseline levels in complementary analyses did not change results (interaction age-by-stress: $p = .014$). To address the direction of the observed group difference we calculated post hoc tests. We found that independent of confounders, the older group displayed an increase in Hcy levels in response to stress (main effect stress: $F(2.5, 39.8) = 3.86$, $p = .022$, $\eta^2 = .19$, $f = 0.48$), while Hcy levels in the younger group did not significantly change (main effect stress: $F(1.3, 29.3) = 1.31$, $p = .27$, $\eta^2 = .06$). As depicted in Fig. 2 in the older group Hcy plasma levels where highest immediately after stress and approached baseline levels during the recovery period.

Notably, overall (i.e. in the combined total group), psychosocial stress did not significantly change Hcy levels independent of age, BMI, and MAP (p=.32) even when additionally controlling for Hcy confounders (p=.53).

Please insert figure 2 around here.

Discussion

To our knowledge, this is the first study to investigate whether Hcy responsiveness to acute stress differs between older and younger men. We intentionally selected from an existing sample of apparently healthy men aged between 20 to 65 yrs a group of younger men without age-related CVD risk (age range 20-30) and a group of middle-aged to older men with age-related CVD risk (age range 47-65 yrs)(Roger et al., 2011). We found an increase in plasma Hcy immediately after stress in the older group, but none in the younger group. In other words, while the older group showed Hcy stress reactivity, Hcy levels in the younger group did not significantly change. Associations were of medium to large effect size and independent of CVD risk factors, Hcy confounders, and Hcy baseline levels. Furthermore, we found that stress per se did not change Hcy levels independent of age (or age group). Notably, our psychosocial stress protocol induced significant increases in free salivary cortisol in both age groups demonstrating the effectiveness of our stress protocol in triggering a neuroendocrine stress response. While the cortisol stress response was elevated in the older group corroborating previous findings (Kudielka et al., 2004), controlling for total cortisol secretion (by calculating the area under the curve (Pruessner et al., 2003)) did not significantly relate to Hcy stress reactivity (p's > .60) nor did it change any of the reported results on Hcy stress reactivity (data not shown).

What are the potential implications of these findings und how do they relate to the literature? Our data suggest that psychosocial stress does not evoke a Hcy response per se, but

only in interaction with age. Numerous case-control and prospective studies have shown that mildly to moderately elevated plasma Hcy levels are positively associated with atherothrombotic vascular disease in the coronary (Lee et al., 2003), cerebral (Perry et al., 1995; Eikelboom et al., 2000), and peripheral arteries (Graham et al., 1997; Humphrey et al., 2008). A previous meta-analysis of prospective studies found that 25% (or, typically, 3 μmol/L) lower plasma Hcy is associated with an 11% lower risk of coronary heart disease and a 19% lower risk of stroke (2002). In our dataset, mean Hcy increase in response to stress in the older group was 2 μmol/L, with a highest observed absolute increase of 4.8 μmol/L. Given these increase levels, the findings of our study possibly point to a mechanism by which mental stress increases CVD risk in older individuals. However, it has not yet been investigated whether short-term Hcy increases as observed in our study relate to CVD and CVD risk. Moreover, despite in the older group increased Hcy levels in response to stress approached baseline levels in the course of the recovery period, it might be speculated that stress-induced transient elevations in Hcy levels may accumulate in the long term and contribute to higher basal plasma Hcy levels in older individuals. However, further studies are needed to determine whether in older persons increased Hcy levels following stress might contribute to elevated basal plasma Hcy levels.

The hitherto published studies on Hcy stress reactivity are controversial, as a Hcy increase in response to stress could not always be detected. Our study provides first indications that aging affects the Hcy response to stress. In light of this and upon closer observation of the literature two aspects may predict whether a stress-induced increase in Hcy levels occurs or not: the age of the individual and the duration of stress exposure. While Stoney (1999) was able to detect a Hcy increase in middle-aged to older women between 40 and 64 years of age after a 10-minute stress exposure, Farag and colleagues (2003) were unable to replicate this finding in women of a similar age group as well as in younger women

aged between 21 and 41 years after only six minutes of stress exposure. Sawai and colleagues (2008), on the other hand, found that young men between 22 and 26 years of age showed an increase in Hcy after 15 minutes of stress exposure. In contrast, our younger age group showed no increase in Hcy after 10 minutes of stress exposure, while men between 40 and 65 years of age showed an Hcy increase after 10 minutes of stress exposure. Notably, the studies used mental stressors of mild to moderate (e.g. arithmetics only) or stronger (arithmetics plus public speaking) stress reactivity inducing capacity.

What are the underlying mechanisms of the observed Hcy stress increase in the older group? It is unlikely that the observed Hcy increase is a concomitant phenomenon of stress-hemoconcentration (Austin et al., 2011) as the molecular weight of Hcy (0.135 kDa) is substantially smaller than the critical molecular weight of 69 kDa. We can only speculate that underlying mechanisms may include altered Hcy metabolism regulation (Williams and Schalinske, 2007) or altered sensitivity to stress hormone release (Rohleder et al., 2002) with age. We further speculate that the observed age effects on Hcy stress reactivity might be linked to norepinephrine/epinephrine levels which are released in response to acute stress from the adrenal medulla into circulation. Our speculation is based on the fact that Hcy synthesis is promoted by s-adenosylmethionine-dependent methylation of norepinephrine to form epinephrine (Gellekink et al., 2007). However, experimental studies are needed to determine exactly whether norepinephrine or epinephrine play a role in Hcy stress reactivity in different age groups.

Our study has several strengths, including recruitment of apparently healthy and unmedicated subjects with reasonable health habits. This is important, because blood Hcy is affected by numerous lifestyle factors and drugs (Refsum et al., 1998; Castro et al., 2006). Furthermore, we statistically controlled for the potential confounding factors BMI, MAP,

vitamin B6, folate, and creatinine. We also used a highly standardized and potent stress test that reliably induces neuroendocrine stress responses (Dickerson and Kemeny, 2004).

However, the study also has its limitations. First of all, the clinical relevance of the observed short-term Hcy increases with older age needs to be investigated in future research. Moreover, although our study provides first indications that aging affects stress reactivity of Hcy, based on our sample, no inference can be made at what age Hcy stress reactivity starts to increase. Large-scale studies are needed to address these questions. Furthermore, the underlying mechanisms of the observed age effect remain to be elucidated and Hcy reactivity in interaction with age should also be investigated following repeated acute stress induction. Moreover, our findings were obtained in a sample of apparently healthy men and may not be generalized to individuals with overt CVD or women.

Taken together, we found first indications that age affects the Hcy response to stress. We observed Hcy stress reactivity in middle-aged to older but not in younger men. Given the association of mild to moderate hyperhomocysteinemia with atherogenesis and thrombogensis, our findings possibly point to a mechanism by which mental stress increases CVD risk in older individuals. However, the clinical relevance of our findings needs to be investigated and future studies are needed to replicate our findings in larger samples with broader age ranges. Moreover, the underlying mechanism of Hcy increases following stress with older age remains to be elucidated.

References

2002. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA 288, 2015-2022.

Austin, A.W., Patterson, S.M., von Kanel, R., 2011. Hemoconcentration and hemostasis during acute stress: interacting and independent effects. Ann Behav Med 42, 153-173.

Black, P.H., Garbutt, L.D., 2002. Stress, inflammation and cardiovascular disease. J Psychosom Res 52, 1-23.

Bogdanski, P., Pupek-Musialik, D., Dytfeld, J., Lacinski, M., Jablecka, A., Jakubowski, H., 2008. Plasma homocysteine is a determinant of tissue necrosis factor-alpha in hypertensive patients. Biomed Pharmacother 62, 360-365.

Brotman, D.J., Golden, S.H., Wittstein, I.S., 2007. The cardiovascular toll of stress. Lancet 370, 1089-1100.

Casas, J.P., Bautista, L.E., Smeeth, L., Sharma, P., Hingorani, A.D., 2005. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. Lancet 365, 224-232.

Castro, R., Rivera, I., Blom, H.J., Jakobs, C., Tavares de Almeida, I., 2006. Homocysteine metabolism, hyperhomocysteinaemia and vascular disease: an overview. J Inherit Metab Dis 29, 3-20.

Clarke, R., Bennett, D.A., Parish, S., Verhoef, P., Dotsch-Klerk, M., Lathrop, M., Xu, P., Nordestgaard, B.G., Holm, H., Hopewell, J.C., Saleheen, D., Tanaka, T., Anand, S.S., Chambers, J.C., Kleber, M.E., Ouwehand, W.H., Yamada, Y., Elbers, C., Peters, B., Stewart, A.F., Reilly, M.M., Thorand, B., Yusuf, S., Engert, J.C., Assimes, T.L., Kooner, J., Danesh, J., Watkins, H., Samani, N.J., Collins, R., Peto,

R., 2012. Homocysteine and coronary heart disease: meta-analysis of MTHFR case-control studies, avoiding publication bias. PLoS Med 9, e1001177.

Clarke, R., Halsey, J., Lewington, S., Lonn, E., Armitage, J., Manson, J.E., Bonaa, K.H., Spence, J.D., Nygard, O., Jamison, R., Gaziano, J.M., Guarino, P., Bennett, D., Mir, F., Peto, R., Collins, R., 2010. Effects of lowering homocysteine levels with B vitamins on cardiovascular disease, cancer, and cause-specific mortality: Meta-analysis of 8 randomized trials involving 37 485 individuals. Arch Intern Med 170, 1622-1631.

Cronin, S., Furie, K.L., Kelly, P.J., 2005. Dose-related association of MTHFR 677T allele with risk of ischemic stroke: evidence from a cumulative meta-analysis. Stroke 36, 1581-1587.

de Oliveira, A.C., Suchecki, D., Cohen, S., D'Almeida, V., 2004. Acute stressor-selective effect on total plasma homocysteine concentration in rats. Pharmacol Biochem Behav 77, 269-273.

de Souza, F.G., Rodrigues, M.D., Tufik, S., Nobrega, J.N., D'Almeida, V., 2006. Acute stressor-selective effects on homocysteine metabolism and oxidative stress parameters in female rats. Pharmacol Biochem Behav 85, 400-407.

Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol Bull 130, 355-391.

Dragani, A., Falco, A., Santilli, F., Basili, S., Rolandi, G., Cerasa, L., Lattanzio, S., Ciabattoni, G., Patrono, C., Davi, G., 2012. Oxidative stress and platelet activation in subjects with moderate hyperhomocysteinaemia due to MTHFR 677 C -->T polymorphism. Thromb Haemost 108, 533-542.

Eikelboom, J.W., Hankey, G.J., Anand, S.S., Lofthouse, E., Staples, N., Baker, R.I., 2000. Association between high homocyst(e)ine and ischemic stroke due to large- and

small-artery disease but not other etiologic subtypes of ischemic stroke. Stroke 31, 1069-1075.

Ellinson, M., Thomas, J., Patterson, A., 2004. A critical evaluation of the relationship between serum vitamin B, folate and total homocysteine with cognitive impairment in the elderly. J Hum Nutr Diet 17, 371-383.

Farag, N.H., Barshop, B.A., Mills, P.J., 2003. Effects of estrogen and psychological stress on plasma homocysteine levels. Fertility and sterility 79, 256-260.

Gellekink, H., Muntjewerff, J.W., Vermeulen, S.H., Hermus, A.R., Blom, H.J., den Heijer, M., 2007. Catechol-O-methyltransferase genotype is associated with plasma total homocysteine levels and may increase venous thrombosis risk. Thromb Haemost 98, 1226-1231.

Graham, I.M., Daly, L.E., Refsum, H.M., Robinson, K., Brattstrom, L.E., Ueland, P.M., Palma-Reis, R.J., Boers, G.H., Sheahan, R.G., Israelsson, B., Uiterwaal, C.S., Meleady, R., McMaster, D., Verhoef, P., Witteman, J., Rubba, P., Bellet, H., Wautrecht, J.C., de Valk, H.W., Sales Luis, A.C., Parrot-Rouland, F.M., Tan, K.S., Higgins, I., Garcon, D., Andria, G., et al., 1997. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 277, 1775-1781.

Herrera, M.D., Mingorance, C., Rodriguez-Rodriguez, R., Alvarez de Sotomayor, M., 2010. Endothelial dysfunction and aging: an update. Ageing Res Rev 9, 142-152.

Higashi, Y., Kihara, Y., Noma, K., 2012. Endothelial dysfunction and hypertension in aging. Hypertens Res. 35, 1039-1047.

Humphrey, L.L., Fu, R., Rogers, K., Freeman, M., Helfand, M., 2008. Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis. Mayo Clinic 83, 1203-1212.

Jakubowski, H., 2001. Protein N-homocysteinylation: implications for atherosclerosis. Biomed Pharmacother 55, 443-447.

Jakubowski, H., 2004. Molecular basis of homocysteine toxicity in humans. Cell Mol Life Sci 61, 470-487.

Jakubowski, H., 2005. Anti-N-homocysteinylated protein autoantibodies and cardiovascular disease. Clin Chem Lab Med 43, 1011-1014.

Jakubowski, H., Boers, G.H., Strauss, K.A., 2008. Mutations in cystathionine beta-synthase or methylenetetrahydrofolate reductase gene increase N-homocysteinylated protein levels in humans. FASEB J 22, 4071-4076.

Jendricko, T., Vidovic, A., Grubisic-Ilic, M., Romic, Z., Kovacic, Z., Kozaric-Kovacic, D., 2009. Homocysteine and serum lipids concentration in male war veterans with posttraumatic stress disorder. Prog Neuropsychopharmacol Biol Psychiatry 33, 134-140.

Kirschbaum, C., Pirke, K.M., Hellhammer, D.H., 1993. The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28, 76-81.

Kudielka, B.M., Buske-Kirschbaum, A., Hellhammer, D.H., Kirschbaum, C., 2004. HPA axis responses to laboratory psychosocial stress in healthy elderly adults, younger adults, and children: impact of age and gender. Psychoneuroendocrinology 29, 83-98.

Lee, B.J., Lin, P.T., Liaw, Y.P., Chang, S.J., Cheng, C.H., Huang, Y.C., 2003. Homocysteine and risk of coronary artery disease: Folate is the important determinant of plasma homocysteine concentration. Nutrition 19, 577-583.

McCully, K.S., 1996. Homocysteine and vascular disease. Nat Med 2, 386-389.

Papatheodorou, L., Weiss, N., 2007. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 9, 1941-1958.

Perry, I.J., Refsum, H., Morris, R.W., Ebrahim, S.B., Ueland, P.M., Shaper, A.G., 1995. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. Lancet 346, 1395-1398.

Pruessner, J.C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 28, 916-931.

Refsum, H., Ueland, P.M., Nygard, O., Vollset, S.E., 1998. Homocysteine and cardiovascular disease. Annu Rev Med 49, 31-62.

Roberts, S.B., Williamson, D.F., 2002. Causes of adult weight gain. J Natr 132, 3824S-3825S.

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Greenlund, K.J., Hailpern, S.M., Heit, J.A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Makuc, D.M., Marcus, G.M., Marelli, A., Matchar, D.B., McDermott, M.M., Meigs, J.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G., Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-Rosett, J., 2011. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. Circulation 123, e18-e209.

Rohleder, N., Kudielka, B.M., Hellhammer, D.H., Wolf, J.M., Kirschbaum, C., 2002. Age and sex steroid-related changes in glucocorticoid sensitivity of pro-inflammatory cytokine production after psychosocial stress. J Neuroimmunol 126, 69-77.

Saposnik, G., Ray, J.G., Sheridan, P., McQueen, M., Lonn, E., 2009. Homocysteine-lowering therapy and stroke risk, severity, and disability: additional findings from the HOPE 2 trial. Stroke 40, 1365-1372.

Sawai, A., Ohshige, K., Kura, N., Tochikubo, O., 2008. Influence of mental stress on the plasma homocysteine level and blood pressure change in young men. Clin Exp Hypertens 30, 233-241.

Selhub, J., Jacques, P.F., Wilson, P.W., Rush, D., Rosenberg, I.H., 1993. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 270, 2693-2698.

Smulders, Y.M., Blom, H.J., 2011. The homocysteine controversy. J Inherit Metab Dis 34, 93-99.

Stoney, C.M., 1999. Plasma homocysteine levels increase in women during psychological stress. Life Sci 64, 2359-2365.

Torregrossa, A.C., Aranke, M., Bryan, N.S., 2011. Nitric oxide and geriatrics: Implications in diagnostics and treatment of the elderly. JGC 8, 230-242.

Undas, A., Brozek, J., Jankowski, M., Siudak, Z., Szczeklik, A., Jakubowski, H., 2006. Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. Arterioscler Thromb Vasc Biol 26, 1397-1404.

Wald, D.S., Law, M., Morris, J.K., 2002. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 325, 1202.

Wang, X., Qin, X., Demirtas, H., Li, J., Mao, G., Huo, Y., Sun, N., Liu, L., Xu, X., 2007. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 369, 1876-1882.

Weinsaft, J.W., Edelberg, J.M., 2001. Aging-associated changes in vascular activity: a potential link to geriatric cardiovascular disease. Am J Geriatr Cardiol 10, 348-354.

Williams, K.T., Schalinske, K.L., 2007. New insights into the regulation of methyl group and homocysteine metabolism. J Nutr 137, 311-314.

Wirtz, P.H., Redwine, L.S., Baertschi, C., Spillmann, M., Ehlert, U., von Kanel, R., 2008. Coagulation activity before and after acute psychosocial stress increases with age. Psychosom Med 70, 476-481.

Figure legends

Figure 1

Changes of salivary cortisol to psychosocial stress in younger and middle-aged to older subjects. Values are mean ± SEM. Repeated measures ANOVA revealed significant salivary cortisol increases in response to stress across both age groups (main effect of stress in the total group: $F(2.6/121.6) = 26.82$, $p = <.001$, $\eta^2 = .36$, main effect of stress in the younger group: $F(2.6/67.7) = 11.73$, $p < .001$, $\eta^2 = .31$; main effect of stress in the older group: $F(1.6/32.3) = 26.90$, $p < .001$, $\eta^2 = .57$)).

Figure 2

Changes of plasma homocysteine levels to psychosocial stress in younger and middle-aged to older subjects. Values are mean ± SEM. Repeated measures ANCOVA revealed that the two age groups differed in Hcy stress reactivity ($p = .014$). More precisely, as shown by post hoc tests a Hcy increase following stress could be observed in the older ($p = .022$) but not in the younger group ($p = .27$). Body mass index, mean arterial pressure, vitamin B12, folate, and creatinine were controlled as covariates.

Tables

Table 1 Characteristics in 50 subjects by age categorization

	Younger group (n = 28)	Older group (n = 22)	P-ANOVA
Age (yr)	24.7±0.5 (20-30)	55.8±1.2 (47-65)	<.001
BMI (kg/m^2)	23.2±0.4 (20.7-28.5)	26.3±0.4 (2.4-31.4)	<.001
MAP (mm Hg)	90.4±1.6 (71.2-103.3)	106.9±2.9 (81.7-138.0)	<.001
Hcy (μmol/L)	11.7±1.6 (6.1-41.2)	17.8±3.0 (7.4-47.1)	.033
Vitamine B12 (pg/ml)	493.3±36.6 (229-1012)	446.0±49.4 (295-1424)	.436
Folate (ng/ml)	6.2±0.6 (2.5-15.6)	6.0±0.7 (2.6-15.0)	.807
Creatinine (mg/dl)	1.0±0.02 (0.8-1.3)	1.0±0.03 (0.79-1.4)	.315
Cortisol baseline (nmol/l)	13.3±2.3 (4.3-52.2)	7.7±0.8 (2.1-18.9)	.041

Notes. Values are given as means±SEM. BMI, body mass index; MAP, mean arterial pressure; Hcy, homocysteine.