

Homocysteine as a risk factor for coronary heart diseases and its association with inflammatory biomarkers, lipids and dietary factors

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Abstract

The causal relation of total Homocysteine (tHcy) to coronary heart diseases (CHD) is unclear. In vitro studies suggest a proinflammatory effect. Among 32,826 women from the Nurses' Health Study who provided blood samples in 1989–1990, 237 CHD events were documented during 8 years of follow-up. The cases (1:2) were matched to controls on age, smoking, and month of blood draw. Plasma tHcy was inversely associated with blood levels of folate (partial $r = -0.3$, $P < 0.0001$) and B₁₂ ($r = -0.2$, $P < 0.0001$) and with dietary intake of folate ($r = -0.1$, $P < 0.01$) and B₂ vitamin ($r = -0.1$, $P = 0.01$). tHcy was positively associated with soluble tumor necrosis receptor (sTNF-R) 1 and 2 (partial $r = 0.2$, $P < 0.0001$). In a multivariate model adjusted for age, smoking, BMI, parental history, hypertension, diabetes, postmenopausal hormone use, physical activity and alcohol intake, the relative risk of CHD between the extreme quartiles of tHcy was 1.66 (95% CI: 1.05–2.64, P trend = 0.02). The association was not appreciably attenuated after further adjustments for sTNF-R1, sTNF-R2, CRP, or Total Cholesterol:HDL-c ratio. tHcy is an independent risk predictor of CHD and modestly associated with TNF-receptors. However, the inflammatory biomarkers measured could not explain its role in CHD.

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1. Background

tHcy, a highly reactive sulfur-containing amino acid, is an intermediary product of methionine metabolism which can be either remethylated to methionine or metabolized to cysteine. tHcy is a modest independent predictor of CHD [1,2], but the pathophysiologic mechanism is unclear. tHcy might induce atherosclerosis by impairing coronary microvascular dilator function [3], by smooth muscle proliferation [4], platelet activation, thrombogenesis [5], endothelial dysfunction, and collagen synthesis [6].

In vitro and in vivo studies suggest that tHcy is a potent inducer of inflammation [7] and is involved with inflamma-

Abbreviations: tHcy, total homocysteine; CHD, coronary heart disease; sTNF-R, soluble tumor necrosis factor alpha receptor; IL-6, interleukin 6; BMI, body mass index; CRP, C-reactive protein; C/HDL-c, cholesterol/high density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; MI, myocardial infarction; MTHFR, 5,10-methylene-tetrahydrofolate reductase; ROC, receiver operator characteristic; RR, relative risk; CI, confidence interval; Lp(a), lipoprotein (a); HDL-c, high density lipoprotein cholesterol; CV, coefficient of variation; PLP, pyridoxal 5'-phosphate; FFQ, food frequency questionnaire; NHS, Nurses' Health Study

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tory functions of endothelial cells at the level of gene expression [8]. Elevated levels of tHcy increase IL-6 production in monocytes [9], upregulate vascular cell adhesion molecules, and enhance monocyte adhesion [10]. However, studies with cell culture typically use higher concentrations (mmolar) of freshly prepared tHcy and may not be applicable for human plasma tHcy concentrations (micromolar). In murine models, tHcy enhances inflammation markers [11]. Hyperhomocysteinemic mice show high plasma levels of the inflammatory cytokine TNF α [12] which suggests a proinflammatory effect of tHcy. Epidemiologic studies on associations between tHcy and inflammation are sparse and inconsistent. CRP was not associated with tHcy in CHD [13], hemodialysis [14] or psychogeriatric [15] patients and was positively associated with tHcy in diabetic CHD patients [16].

In a prospective nested case-control study of CHD among middle-aged women in the Nurses' Health Study (NHS), we examined the role of tHcy in CHD and its association with inflammatory biomarkers, lipids, B vitamins in blood and diet and the 5,10-methylene-tetrahydrofolate reductase (MTHFR_{C677T}) polymorphism.

2. Materials and methods

2.1. The Nurses' Health Study (NHS) cohort population and blood collection

The NHS was initiated in 1976, with the enrollment of 121,700 female nurses aged 30–55. Since then, follow-up questionnaires have been mailed to the cohort every 2 years to update information on exposures and the occurrence of major illnesses. Between 1989 and 1990, blood samples were collected from 32,826 women. Within 24 h of collection 97% of the samples were returned. They were immediately centrifuged, aliquoted into plasma, red blood cell, and buffy-coat fractions, and stored in liquid nitrogen.

2.2. Assessment of CHD endpoints

The endpoint for this study comprised incident cases of nonfatal MI and fatal CHD that occurred after the blood collection and before May 31, 1998. Subjects with a previous report of cancer or CHD before the blood collection were excluded. Cases were confirmed if they met the diagnostic criteria of the World Health Organization (i.e., symptoms plus either cardiac enzyme level elevations or diagnostic ECG changes) [17]. Physicians reviewed medical records blinded to exposure status. An MI was defined as probable if medical records were not available but hospitalization occurred, and confirmatory information was obtained by interview or letter. More than 98% of deaths were identified by systematic searches of the state vital records and the National Death Index [18]. Fatal CHD was defined as fatal MI confirmed by hospital records or at autopsy or as CHD recorded on the death certificate, if this was the underlying and most proba-

ble cause given and there was previous evidence of CHD. In no instance was the cause on the death certificate accepted without corroboration. Total CHD was defined as nonfatal MI plus fatal CHD. Each case was matched by year of age, year and month of blood draw, fasting status before blood draw (lower/higher than 8 h) and smoking status (never, past, current) to two controls who were free of CHD at the time of the case diagnosis.

2.3. Ascertainment of diet

Diet was ascertained in 1990 from a validated [19] FFQ. Food composition values for folate, riboflavin, vitamin B6, vitamin B12, and other nutrients were obtained from the Harvard University Food Composition Database (archived version: November 22, 1993) derived from US Department of Agriculture sources [20] and supplemented with food manufacturers' information. The data also gathered information on folate-fortified foods, use of vitamin supplements, and brand and type of multiple vitamins.

2.4. Blood collection and assessment of biomarkers

Measurements of plasma tHcy, folate, vitamin B12 and PLP (the active form of vitamin B₆) have been described previously [21]. In brief, tHcy was determined by HPLC; PLP, by a radiometric tyrosine assay; and folate and B12, by RIA. The average intra-assay CV for folate, vitamin B12, and tHcy were 6.8%, 3.0%, and 2.9%, respectively. RBC folate (folate per gram of hemoglobin) was measured with an automated chemiluminescence system (Bayer Diagnostics, Tarrytown, NY) with average CV of 5.1%. High-sensitivity CRP was measured by an immunoturbidimetric assay on the Hitachi 911 analyzer, with a CV of 1.4%. IL-6 and soluble TNF receptors 1 and 2 (sTNF-R1 and sTNF-R2) were measured by an ultrasensitive ELISA assay (R&D Systems, Minneapolis, MN), with CVs of 10.3%, 6.1%, and 4.1%, respectively. Methods for genotyping the MTHFR_{C677T} polymorphism have previously been published [22].

Total cholesterol was measured enzymatically [23], with a CV of 1.7%. Concentrations of triglycerides and HDL-c were analyzed simultaneously on the Hitachi 911, with CVs of 1.7%, 1.8%, and 2.5%, respectively. LDL-c was determined by a homogenous direct method (Genzyme, Cambridge, MA), with a CV lower than 3.1%. The apolipoprotein B assay was performed by an immunoturbidimetric technique on the Hitachi 911 analyzer, with a CV of 4.3%. Lp(a) was determined by a latex-enhanced immunoturbidimetric method (Denka Sieken, Tokyo Japan) with a CV of 2.6%.

2.5. Data analysis

All nutrient values derived from the FFQ included supplements and, except for alcohol and coffee, were energy-adjusted [24]. We compared baseline characteristics of cases and controls using Student's *t*-test for continuous variables

and the Chi-square test for categorical variables. Because the distribution of tHcy levels was right-skewed, the nonparametric Wilcoxon signed rank test was performed on median plasma values. We used a general linear model to determine the geometric mean level of tHcy after adjusting for matching factors, outcome status (case/control), and BMI. Quartile cut-points were defined according to the distribution of tHcy among controls. We computed RRs and 95% CIs of CHD across tHcy quartiles with a multiple logistic regression and fit the median of each quartile as a continuous variable. We assessed the ability of the models to discriminate events from non-events with the C statistic, which is analogous to the area under the ROC curve [25].

3. Results

3.1. Population characteristics and MTHFR_{C677T} polymorphism

After excluding 24 subjects who were taking cholesterol-lowering drugs at time of blood collection and 9 whose tHcy data were missing, we documented 237 incident MI cases (202 nonfatal MI and 35 fatal CHD) during 8 years of follow-up. Compared with 458 controls, case subjects had higher plasma tHcy levels ($P = 0.02$) were more likely to have diabetes, hypertension, higher BMI and a parental history of MI. In our population, the prevalence of heterozygosity and homozygosity of the thermolabile MTHFR_{C677T} mutation was

44% and 10%, respectively; we observed no association of this mutation to CHD risk (Table 1).

3.2. Homocysteine in relation to blood biomarkers and dietary factors

After controlling for age, BMI, smoking, and outcome status, we explored the association of tHcy with several plasma biomarkers and dietary components. We observed inverse associations ($P < 0.0001$) between tHcy levels (Fig. 1a) and plasma or RBC folate levels ($r = -0.4$), plasma B12 ($r = -0.3$), and PLP ($r = -0.2$). After further adjustment for each of the other plasma B vitamins or RBC folate, the associations of tHcy with RBC folate ($r = -0.3$) and plasma B12 ($r = -0.2$) were slightly attenuated but remained significant ($P < 0.0001$). In contrast, the association of tHcy with PLP was reduced and no longer significant. The cytokines sTNF-R1 and sTNF-R2 were significantly ($r = 0.2$, $P < 0.0001$) associated with elevated tHcy levels (Fig. 1b), but IL-6 and CRP were not (Fig. 1b). However, tHcy levels in the top quartile of IL-6 tended to be higher, as compared to the lower quartile ($P = 0.08$). None of the lipid biomarkers were significantly related to tHcy levels (Fig. 1c). tHcy was inversely associated with dietary intake of folate B2, B6, and B12 vitamins and was positively associated with alcohol intake (Fig. 1d). However, after simultaneously controlling for all dietary variables above, only the association between tHcy to dietary folate ($r = -0.13$, $P = 0.01$) and B2 vitamin ($P = -0.12$, $P = 0.01$) persisted. Consumption of at least 2 cups of coffee a day was

Table 1
Baseline clinical characteristics of 237 incident CHD cases and 458 controls from the Nurses Health Study (1990–1998)

Variable ^a	CHD cases ($n = 237$)	Controls ($n = 458$)	P value	Relative risk (95% CI) ^b
Age (years \pm S.D.)	61.4 \pm 6.6	61.2 \pm 6.6	Matched	
Smokers (%)			Matched	
Never	34.6	34.2		
Past	35.0	35.4		
Current	30.4	30.4		
Body mass index (kg/m ² , %)				
≤ 25	43.9	53.1	<0.001	1
>25 to ≤ 30	23.2	26.6		0.83 (0.57–1.20)
>30	32.9	20.3		1.95 (1.36–2.79)
Parental MI before age 60 (%)	21.9	12.5	<0.001	2.01 (1.33–3.05)
History of hypertension (%)	37.6	19.4	<0.001	2.53 (1.77–3.60)
History of diabetes (%)	13.9	2.8	<0.001	5.63 (2.89–10.96)
Menopausal status (%)	90.3	87.2	0.24	1.53 (0.76–3.04)
Postmenopausal hormone use (%)	30.0	36.0	0.10	0.76 (0.54–1.06)
MTHFR (%)				
Ala–Ala (wild type)	44.7	47.4	0.99	1
Ala–Val	45.6	42.9		1.13 (0.81–1.58)
Val–Val (variant)	9.7	9.7		1.06 (0.60–1.87)
Homocysteine (μ mol/l) ^c				
Median	10.6	10.0	0.002	
(inter-quartile)	8.7–12.9	8.2–12.3		

^a \pm S.D. for means.

^b Adjusted for age and smoking.

^c Nonparametric Wilcoxon signed rank test.

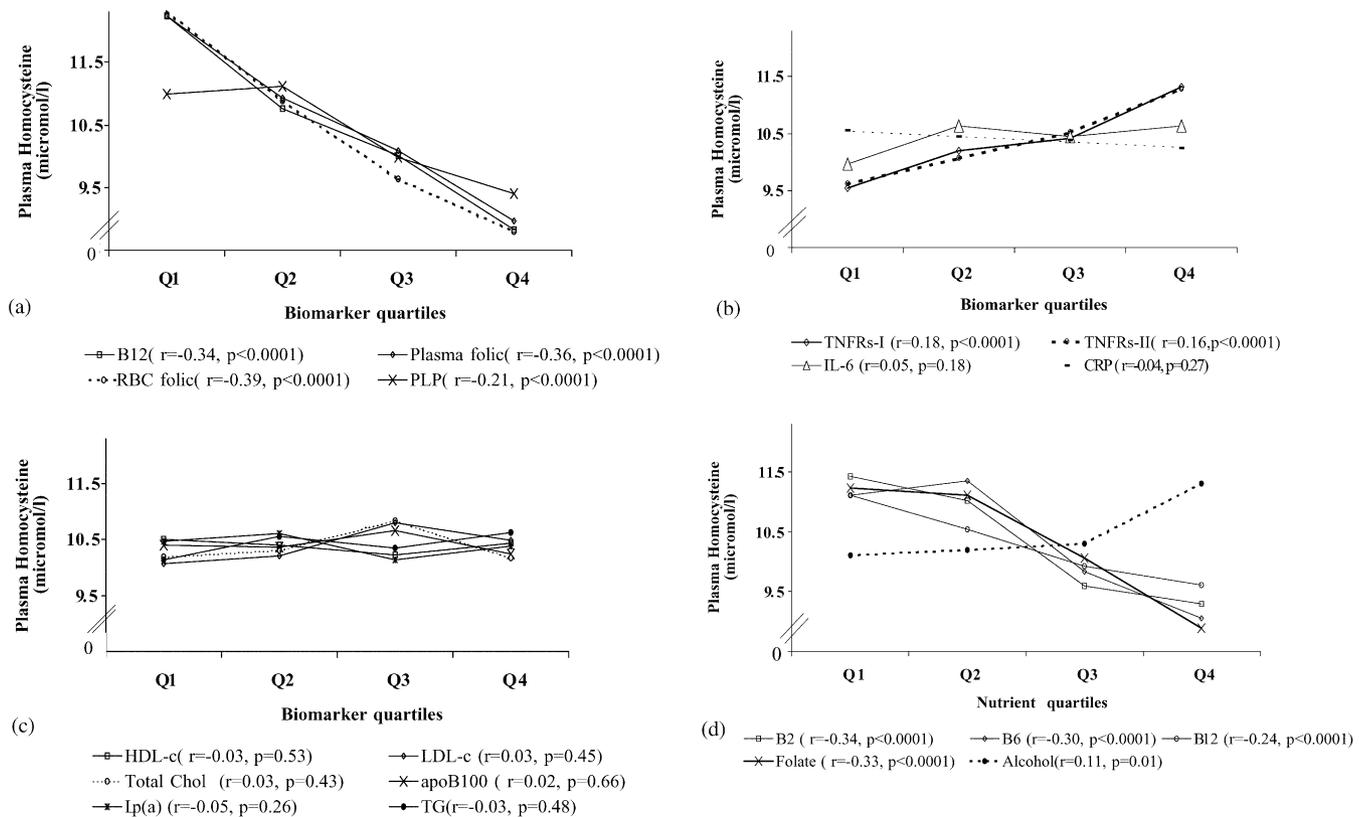


Fig. 1. Multivariable adjusted (adjusted for age, BMI, smoking, and CHD outcome groups) homocysteine levels (homocysteine geometric mean, $\mu\text{mol/l}$) by quartiles (quartiles defined according to the non-cases blood level cut-points: B₁₂: 323.3, 420.1, 8536.2 (pg/ml); RBC folic: 943.9, 1177.8, 1420.9 (ng/gHb); plasma folic: 4.4, 7.1, 11.2 (ng/ml); PLP: 26.5, 41.3, 70.1 (pmol/ml); CRP: 0.1, 0.22, 0.53 (mg/dl); sTNF-R1: 1034.3, 1217.5, 1439.6 (pg/ml); sTNF-R2: 1975.7, 2361.1, 2857.8 (pg/ml); IL-6: 1.1, 1.6, 2.5 (pg/ml); HDL: 48.0, 58.7, 69.6 (mg/dl); LDL: 105.9, 132.4, 155.0 (mg/dl); apoB₁₀₀: 92.1, 111.0, 136.3 (mg/dl); Lp(a): 4.2, 8.7, 21.5 (mg/dl); triglycerides: 74, 110, 149 (mg/dl); Dietary: B2: 1.6, 2.1, 3.9 (mg/day); B6: 1.8, 2.4, 4.3 (mg/day); B12: 6, 8, 11.5 (ng/day); Folate: 270, 361.5, 533 (ng/day); alcohol: 0, 1.8, 8.5 (g/day)) of biomarkers and nutrients intake among the Nurses Health Study' women; (a) plasma B vitamins and red blood cell folate; (b) Inflammatory biomarkers; (c) Lipid biomarkers; (d) Dietary intake.

related to higher tHcy levels (10.7 versus 10.2 $\mu\text{mol/l}$, $P = 0.05$, adjusted for age, BMI, smoking, and outcome status). Similar associations observed in an analysis restricted to the control group.

3.3. Relative risk of CHD across quartiles of Homocysteine levels

The RR of CHD comparing the extreme quartiles of tHcy (Table 2) was 1.58 (95% CI; 1.03–2.44) when adjusting for age, smoking and blood draw parameters. The association persisted in a multivariate model adjusted for age, smoking, BMI, parental history, hypertension, diabetes, postmenopausal hormone use, physical activity, blood draw parameters, and alcohol intake [RR = 1.66 (95% CI; 1.05–2.64)]. The association was not appreciably altered after further adjustments for sTNF-R1, sTNF-R2, CRP, or C/HDL-c ratio. In a multivariate model incorporating the established CHD risk factors [26] C/HDL-c ratio and CRP, the RR of CHD between the extreme quartiles of tHcy was 1.56 (95% CI; 0.97–2.49, P trend = 0.03) and the calculated area under the ROC curve was 0.72. Since estrogen levels may re-

duce tHcy concentrations [27], we performed the regression analyses with and without including current postmenopausal hormone use and the models yielded similar results.

4. Discussion

In a nested prospective case-control assessment including 202 nonfatal MI and 35 fatal CHD events during 8 years of follow-up, we found a positive association between tHcy levels and CHD risk. We observed a linear trend, but the elevated risk was significant only in the top quartile. This association persisted after controlling for other established CHD factors. tHcy levels were independently inversely correlated with elevated blood levels and dietary intake of folate, and modestly associated directly with sTNF-receptor levels.

With respect to short-term stability, analysis of blood samples from the NHS showed that the levels of biomarkers measured in this study, including the inflammatory biomarkers were stable for up to 36 h from collection until processing [28]. Previous studies have documented the effect of folate, PLP, and vitamin B12 on tHcy levels [29], supporting the validity of our plasma markers. However, although a single

Table 2
Relative risks (95% CI) of CHD across quartiles of homocysteine

	Q ₁	Q ₂	Q ₃	Q ₄	P trend	Area under ROC ^a
Median tHcy (μmol/l) ^b	7.2	9.2	10.9	14.4		
Model 1 ^c	1	1.05 (0.65–1.70)	1.25 (0.71–2.22)	1.58 (1.03–2.44)	0.02	0.57
Model 2 ^d Multivariable (MV)	1	1.12 (0.67–1.87)	1.35 (0.74–2.47)	1.66 (1.05–2.64)	0.02	0.69
MV + C:/HDL-c ratio	1	1.04 (0.62–1.75)	1.26 (0.68–2.34)	1.55 (0.97–2.49)	0.03	0.72
MV + sTNF-R1	1	1.08 (0.65–1.79)	1.29 (0.70–2.37)	1.56 (0.98–2.50)	0.04	0.69
MV + sTNF-R2	1	1.05 (0.63–1.76)	1.27 (0.69–2.34)	1.54 (0.96–2.47)	0.04	0.69
MV + CRP	1	1.12 (0.67–1.86)	1.36 (0.74–2.49)	1.66 (1.05–2.65)	0.02	0.69
MV + C:/HDL-c ratio + CRP		1.03 (0.62–1.74)	1.27 (0.69–2.36)	1.56 (0.97–2.49)	0.03	0.72

^a ROC – the receiver operating characteristic.

^b Cut-points according to the distribution in the control group.

^c Model 1: adjusted for age, smoking (never, past, current), fasting hours before blood draw (</>8 h), problems at blood drawing (no problems, more than one day since drawn, moderate hemolysis), and tHcy batch samples (from 1990–1994, 1996, 1998).

^d Model 2: adjusted model 1: variables + BMI (<25, >25 to <30, >30), parental MI before age 60, hypertension, diabetes, postmenopausal hormone use, physical activity (metabolic equivalent hours, quartiles) and alcohol intake (quartiles).

measurement of tHcy can be used to characterize the average tHcy concentration [30], the intra-individual variation over time for inflammatory biomarker levels [31] may attenuate the associations observed over 8-year follow-up. In order to overcome variability in time, we controlled for tHcy sample batches. The generalizability of these findings, from a cohort of female nurses, may be questioned. However, the interquartile range of tHcy levels (8.2–12.3 μmol/l) in this population is similar to that observed in other group studies [1,2], and the cohort homogeneity in socio-educational parameters may serve to reduce the potential for bias by other unmeasured confounders. The prospective design and high follow-up rates in this study minimize the possibility that our findings are due to methodologic biases. Because controlling for established risk factors for CHD had a minimal effect on the association of tHcy to CHD, our results are unlikely to be explained by residual confounding by those factors.

We found that tHcy in the top quartile, with median levels of 14.4 μmol/l, was associated with 66% increase in risk of CHD as compared with tHcy in the lower quartile, with median levels of 7.2 μmol/l. A recent meta-analysis [1] reported that after adjustment for limited cardiovascular risk factors and intraindividual variability in tHcy levels, a 25% lower usual tHcy level (about 3 μmol/l) was associated with an 11% lower risk of ischemic heart disease (OR = 0.89; 95% CI, 0.83–0.96). A parallel meta-analysis [2] suggested that the association between tHcy and cardiovascular diseases is causal and that lowering tHcy concentrations by 3 μmol/l should reduce the risk of ischaemic heart disease by 16% (11%–20%). However, few studies controlled for comprehensive CHD risk factors in tHcy prediction models. In contrast with findings from the Women's Health Study (*n* = 126 cardiovascular events) [26], the association we observed between tHcy and CHD did not appreciably attenuate after controlling simultaneously for CRP and C:/HDL ratio.

Unlike the strong associations between tHcy and inflammation found in cell cultures, [7–10] and in hyperhomocysteinemic mice models, [11,12] we found only a modest but consistent association between the upstream

signaling products sTNF-receptors in relation to tHcy. sTNF-receptors are derived by proteolytic cleavage from TNF cell surface receptors after induction by TNF or other cytokines such as IL-6, IL-1β, or IL-2 and have a longer half-life and are detected with a higher sensitivity than TNF [32]. sTNF-receptors are indicators of inflammatory processes [32], whereas CRP is regulated by different cytokines, particularly IL-6. However, we did not observe the same associations with IL-6, although levels of tHcy in the top quartile of IL-6 tended to be higher. tHcy has been suggested to initiate a cascade of inflammatory pathways by oxidant stress [33], leading to endothelial lesions or by recruitment of mononuclear cells to sites of endothelial injury, resulting in the prolonged inflammatory response typical of atherosclerotic lesions [8]. TNF-α is a major factor modulating inflammatory responses in the endothelium exposed to stimulating agents. Although tHcy was significantly associated with sTNF-receptors, our data do not suggest a causal link, since sTNF-receptors nor other inflammatory biomarkers measured, attenuated the association of tHcy to CHD. In a separate analyses, we did find that similar to previous studies [34], sTNF-receptors and other inflammatory markers were associated with risk of CHD [35]. However, our study is limited in that only 10% of our population had tHcy levels greater than exceed 15 μmol/l.

MTHFR_{C677T} is necessary for the remethylation of tHcy to methionine. A common genetic polymorphism that codes for MTHFR is associated with modestly higher tHcy levels. A recent meta-analysis [36] noted that subjects with the MTHFR variant genotype had a 16% (95% CI; 5%, 28%) greater risk of CHD than subjects with the wild-type genotype. We did not find a positive association in our study for the MTHFR homozygosity, but our sample size was not adequate to detect such a modest relationship (as indicated by the wide CI).

In the general population, the most important modifiable determinants of tHcy are dietary folate, B vitamins and coffee consumption [37]. Our results reflect the beneficial effect of intake of folate and B vitamins on reducing tHcy levels. Folate

intake has previously been found to be important determinant of reduced in the primary prevention of CHD in the NHS [38]. Randomized controlled trials of tHcy-lowering vitamins for cardiovascular endpoints [39,40] may clarify whether tHcy is causative in the pathogenesis of atherosclerosis or is simply related to other confounding cardiovascular risk factors. Coffee intake above 2 cups/day was related to higher levels of tHcy. This association was also found in clinical trials [41], but the mechanism of action is unclear. The association of alcohol to the impairment of folate absorption [42] moderates the direct association of alcohol to tHcy. However, overall, alcohol has a significant protective role in CHD [43] and alcohol consumers are often advised to maintain a high folate intake.

We found that tHcy is an independent predictor of CHD. tHcy is modestly associated with sTNF-receptors, but the association of tHcy with risk could not be explained by inflammation.

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References

- [1] The Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002;288:2015–22.
- [2] Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* 2002;325:1202–9.
- [3] Tawakol A, Forgiione MA, Stuehlinger M, et al. Homocysteine impairs coronary microvascular dilator function in humans. *J Am Coll Cardiol* 2002;40:1051–81.
- [4] Tang L, Mamotte CDS, Van Bockxmeer FM, et al. The effect of homocysteine on DNA synthesis in cultured human vascular smooth muscle. *Atherosclerosis* 1998;136:169–73.
- [5] Rodgers GM, Kane WH. Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. *J Clin Invest* 1986;77:1909–16.
- [6] Tsai JC, Perrella MA, Yoshizumi M, et al. Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. *Proc Natl Acad Sci USA* 1994;91:6369–73.
- [7] McCully KS, Vezeridis MP. Histopathological effects of homocysteine thiolactone on epithelial and stromal tissues. *Exp Mol Pathol* 1989;51:159–70.
- [8] Roth J, Goebeler M, Ludwig S, et al. Homocysteine inhibits tumor necrosis factor-induced activation of endothelium via modulation of nuclear factor-kappa b activity. *Biochim Biophys Acta* 2001;1540:154–65.
- [9] Van Aken BE, Jansen J, van Deventer SJ, et al. Elevated levels of homocysteine increase IL-6 production in monocytic Mono Mac 6 cells. *Blood Coagul Fibrinolysis* 2000;11:159–64.
- [10] Silverman MD, Tumuluri RJ, Davis M, et al. Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. *Arterioscler Thromb Vasc Biol* 2002;22:587–92.
- [11] Hofmann MA, Lalla E, Lu Yan, et al. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest* 2001;107:675–83.
- [12] Janssen-Heininger Y.M.W., Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor. *B Free Rad Biol Med* 2000;28:1317–27.
- [13] Rothenbacher D, Fischer HG, Hoffmeister A, et al. Homocysteine and methylenetetrahydrofolate reductase genotype: association with risk of coronary heart disease and relation to inflammatory, hemostatic, and lipid parameters. *Atherosclerosis* 2002;162:193–200.
- [14] Bayes B, Pastor MC, Bonal J, et al. Homocysteine, C-reactive protein, lipid peroxidation and mortality in haemodialysis patients. *Nephrol Dial Transplant* 2003;18:106–12.
- [15] Nilsson K, Hultberg B, Gustafson L. Lack of association between plasma homocysteine and inflammation in psychogeriatric patients. *Dement Geriatr Cogn Disord* 2002;14:151–5.
- [16] Mojiminiyi OA, Abdella N, Moussa MA, et al. Association of C-reactive protein with coronary heart disease risk factors in patients with type 2 diabetes mellitus. *Diabetes Res Clin Pract* 2002;58:37–44.
- [17] Rose G, Blackburn H. Cardiovascular survey methods. 2nd ed. Geneva: World Health Organization; 1982.
- [18] Stampfer MJ, Willett WC, Speizer FE, et al. Test of the national death index. *Am J Epidemiol* 1984;119:837–9.
- [19] Willett WC, Sampson L, Stampfer MJ, et al. Reproducibility and validity of a semi quantitative food frequency questionnaire. *Am J Epidemiol* 1985;122:51–65.
- [20] US Department of Agriculture. Composition of Foods-Raw, Processed, and Prepared, 1963–1992. Agricultural Handbook Series No. 8. Washington, DC: US Dept of Agriculture; 1993.
- [21] Wu K, Helzlsouer KJ, Comstock GW, et al. A prospective study on folate, B12, and pyridoxal 5'-phosphate (B6) and breast cancer. *Cancer Epidemiol Biomarkers Prev* 1999;8:209–17.
- [22] Chen J, Giovannucci E, Kelsey K, et al. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res* 1996;56:4862–4.
- [23] Allain CC, Poon LS, Chan CSG, et al. Enzymatic determination of total serum cholesterol. *Clin Chem* 1974;20:470–5.
- [24] Willett WC, Stampfer MJ. Total energy intake: implications for epidemiologic analyses. *Am J Epidemiol* 1986;124:17–27.
- [25] Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143:29–36.
- [26] Ridker PM, Hennekens CH, Buring JE, et al. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med* 2000;342:836–43.
- [27] Dimitrova KR, DeGroot K, Myers AK, et al. Estrogen and homocysteine. *Cardiovasc Res* 2002;53:577–88.
- [28] Pai JK, Curhan GC, Cannuscio CC, et al. Stability of novel plasma markers associated with cardiovascular disease: processing within 36 h of specimen collection. *Clin Chem* 2002;48:1781–4.
- [29] Selhub J, Jacques PF, Wilson PW, et al. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* 1993;270:2693–8.
- [30] Rossi E, Beilby JP, McQuillan BM, et al. Biological variability and reference intervals for total plasma homocysteine. *Ann Clin Biochem* 1999;36:56–61.
- [31] Macy EM, Hayes TE, Tracy RP. Variability in the measurement of C-reactive protein in healthy subjects: implications for reference intervals and epidemiological applications. *Clin Chem* 1997;43:52–8.

- [32] Aderka D. The potential biological and clinical significance of the soluble tumor necrosis factor receptors. *Cytokine Growth Factor Rev* 1996;7:231–40.
- [33] Kanani P, Sinkey C, Browning R, et al. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 1999;100:1161–8.
- [34] Lind L. Circulating markers of inflammation and atherosclerosis. *Atherosclerosis* 2003;169(2):203–14.
- [35] Pai JK, Ma J, Hankinson SE, et al. Are C-reactive protein (CRP) and fibrinogen associated with increased risk of MI in traditionally low-Risk women? Abstract 26. *Circulation* 2003;107:7001.
- [36] Klerk M, Verhoef P, Clarke R, et al., MTHFR Studies Collaboration Group. MTHFR 677CT polymorphism and risk of coronary heart disease: a meta-analysis. *JAMA* 2002;288:2023–31.
- [37] De Bree A, Verschuren WM, Kromhout D, et al. Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. *Pharmacol Rev* 2002;54:599–618.
- [38] Rimm EB, Willett WC, Hu FB, et al. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. *JAMA* 1998;279:359–64.
- [39] Schnyder G, Roffi M, Flammer Y, et al. Effect of homocysteine-lowering therapy with folic acid, vitamin B12, and vitamin B6 on clinical outcome after percutaneous coronary intervention: the Swiss Heart study: a randomized controlled trial. *JAMA* 2002;288:973–9.
- [40] Hackam DG, Anand SS. Emerging risk factors for atherosclerotic vascular disease: a critical review of the evidence. *JAMA* 2003;290(7):932–40.
- [41] Verhoef P, Pasman WJ, Van Vliet T, et al. Contribution of caffeine to the homocysteine-raising effect of coffee: a randomized controlled trial in humans. *Am J Clin Nutr* 2002;76(6):1244–8.
- [42] Halsted CH, Villanueva JA, Devlin AM, et al. Metabolic interactions of alcohol and folate. *J Nutr* 2002;132(Suppl 8):2367S–72S.
- [43] Rimm EB, Williams P, Fosher K, et al. Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and haemostatic factors. *BMJ* 1999;319(7224):1523–8.