Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men

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Summary

Moderate hyperhomocysteinaemia is common in the general population and has been linked with cardiovascular disease. However, there are no data from prospective, population-based studies. We examined the association between serum total homocysteine (tHcy) concentration and stroke in a nested case-control study within the British Regional Heart Study cohort.

Between 1978 and 1980 serum was saved from 5661 men, aged 40–59 years, randomly selected from the population of one general practice in each of 18 towns in the UK. During follow-up to December, 1991, there were 141 incident cases of stroke among men with no history of stroke at screening. Serum tHcy was measured in 107 cases and 118 control men (matched for age-group and town, without a history of stroke at screening, who did not develop a stroke or myocardial infarction during follow-up). tHcy concentrations were significantly higher in cases than controls (geometric mean 13·7 [95% CI 12·7–14·8] *vs* 11·9 [11·3–12·6] μmol/L; p=0·004). There was a graded increase in the relative risk of stroke in the second, third, and fourth quarters of the tHcy distribution (odds ratios 1·3, 1·9, 2·8; trend p=0·005) relative to the first. Adjustment for age-group, town, social class, body-mass index, hypertensive status, cigarette smoking, forced expiratory volume, packed-cell volume, alcohol intake, diabetes, high-density-lipoprotein cholesterol, and serum creatinine did not attenuate the association.

These findings suggest that tHcy is a strong and independent risk factor for stroke.

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Introduction

Homocysteine (Hcy—used for both forms, with reduced or oxidised sulphhydryl group) is a sulphur-containing aminoacid produced by the demethylation of methionine. Pronounced elevation of total homocysteine (tHcy—protein-bound plus non-protein-bound) concentrations in the blood, due to rare inherited defects of the enzymes involved in its metabolism, is associated with premature cardiovascular disease in early adolescence and even in childhood.[1–3] Moderately elevated Hcy concentrations, reflecting less severe genetic defects and deficiency of nutritional factors required for Hcy metabolism (folic acid, vitamin B12, vitamin B6) are common in the general population.[4] There are consistent data from more than 20 cross-sectional and case-control studies linking moderate hyperhomocysteinaemia with vascular disease, including peripheral vascular disease, ischaemic heart disease, and stroke.[2] Graded associations are described between plasma tHcy concentration and carotid-artery intimal-medial wall thickening[5] and the prevalence of carotid artery stenosis.[6] Plausible biological mechanisms are adduced to link Hcy with vascular endothelial dysfunction,[7] vascular smooth muscle proliferation,[8] and coagulation abnormalities.[2,9]

There have been few prospective studies of the association between tHcy concentration and cardiovascular disease, and all used a nested case-control design with prospectively collected blood samples. Two prospective studies on the association between tHcy and myocardial infarction[10,11] both found a positive association, although in one[10] the increased risk was confined to the top 5% of the distribution.[10] In the single prospective study on the association between tHcy concentrations and ischaemic stroke a weak non-significant association was observed.[12] Stronger associations of borderline significance were detected in low-risk subgroups (ie, younger men and those with normal blood pressure). In another prospective study of tHcy and atherosclerotic disease in middle-aged men and women in Finland, no association with myocardial infarction or stroke was observed.[13] The number of stroke events in that study was small and the prevalence of hyperhomocysteinaemia in Finland is low. There is a clear need to examine this association in a general population sample with a wide range of Hcy concentrations.

We have carried out a nested case-control study (with prospectively collected blood samples) of the association between serum tHcy concentration and stroke, in a representative sample of middle-aged men in the UK, the British Regional Heart Study (BRHS) cohort.

Subjects and methods

For the BRHS, 7735 men aged 40–59 years were randomly selected (by means of an age-sex register) from one general practice in each of 24 towns in England, Wales, and Scotland between January, 1978, and June, 1980, for a prospective study of cardiovascular disease. The criteria for selecting towns, general practices, and subjects, and details of the respondents and data collection have been described.[14,15] Men with cardiovascular or

other disease and those receiving regular medication were not excluded. The overall response rate was 78%.

Research nurses administered a standard questionnaire and examined each man.[16] The questionnaire included questions on occupation, smoking habits, alcohol intake, medical history, and regular medication, including use of antihypertensive drugs.[14,15,17] Occupational status was classified as non-manual or manual. For this study, men were classified as current smokers or non-smokers and into four groups according to current alcohol intake—none or occasional (<1 unit per week), light (1–15 units per week), moderate (16–42 units per week), and heavy (>42 units per week).[18] Body-mass index was used as an index of relative weight. Blood pressure was recorded with a London School of Hygiene sphygmomanometer; two recordings were taken and the mean was used in the analysis with adjustment for inter-observer variation. Hypertension was defined as systolic pressure of 160 mm Hg or higher, diastolic pressure of 95 mm Hg or higher, use of antihypertensive therapy, or any combination of these criteria. Forced expiratory volume in 1 s (FEV_1) was measured with the subject seated, by a Vitalograph spirometer.

From the WHO (Rose) chest pain questionnaire and a three-orthogonal-lead electrocardiogram, pre-existing coronary heart disease at screening was defined on the basis of any of the following criteria: recall of doctor diagnosis of angina or heart attack; a chest-pain questionnaire response indicating angina or possible myocardial infarction; or electrocardiographic evidence of definite or possible myocardial ischaemia or infarction.[16]

More than 99% of study participants have been followed up for morbidity and mortality to December, 1991, an average of 12·8 years' follow-up. Full details of follow-up procedures and the criteria for fatal and non-fatal stroke events have been described.[17,19] Information on death was obtained through the established flagging procedures provided by the National Health Service registers. Fatal stroke events were those assigned on the death certificate to cerebrovascular heart disease (codes 430–38, International Classification of Diseases, 9th Revision). All death certificates in which coding for stroke appeared inappropriate, or in which stroke was not the attributed code when it might have been, were investigated by correspondence with the certifying doctor and the relevant hospital. Non-fatal stroke events were those that produced a neurological deficit present for longer than 24 h. Death within 28 days of the onset of symptoms led to classification as a fatal episode. Non-fatal strokes were ascertained by means of a postal questionnaire to the men after 5 years of follow-up (with a 98% response rate) and by systematic inspection of primary care records every 2 years between 1980 and 1992.[19]

Samples of serum were saved from 5661 men in 18 of the 24 towns. During follow-up to December, 1991, there were 147 cases of stroke among this group of men, of which 141 were new (incident) cases and were eligible for inclusion in this study. 140 potentially eligible controls were randomly chosen from men who were without a history of stroke at screening, who survived to December, 1991, and who did not have a stroke or myocardial infarction during follow-up. Cases and controls were frequency matched by town and age-group (four 5-year age-bands). All of the samples from one of the 18 towns were missing. Samples for measurement of Hcy were retrieved for 107 cases and 97 controls. 21 additional control samples were obtained from two towns (Guildford and Burnley), to bring the total number of controls to 118. In this population, cardiovascular disease mortality is below average in Guildford and above average in Burnley. The men from whom the extra 21 control samples were obtained were similar to the 97 randomly chosen controls as regards the distribution of major stroke risk factors including hypertension and cigarette smoking. There were slightly fewer than expected manual workers but a higher than expected prevalence of pre-existing CHD. A higher proportion of the missing cases than the available cases were manual workers but few had evidence of pre-existing CHD. The distribution of other stroke risk factors was similar among the missing and available cases.

Variables	Cases (n=107)	Controls (n=118)	p
Demography (mean [SD])			
Age (years)*	54·0 (5·0)	53·6 (4·7)	· ·
Body-mass index (kg/m²)	25·9 (3·4)	25·1 (2·8)	0·05
Lifestyle (% of subjects)			
Manual workers	68·9	47·8	0·002
Current smokers	58·9	33·9	<0·001
Heavy drinkers	16·8	3·4	0·002
History (% of subjects)			
Evidence of CHD	37·4	5·1	<0·001
Hypertensive	67·3	23·7	<0·001
History of diabetes	4·7	0·8	0·10
Clinical characteristics (mean [SD])			
Systolic BP (mm Hg)	165 (25)	141 (17)	<0·001
Diastolic BP (mm Hg)	89 (17)	79 (12)	<0·001
Cholesterol (mmol/L)	6·29 (0·90)	6·40 (1·10)	0·4
HDL-cholesterol (mmol/L)	1·12 (0·27)	1·19 (0·25)	0·05
Packed-cell volume (%)	45·4 (3·8)	44·2 (2·5)	0·01
FEV_1 (mL)	299·4 (74·5)	333·0 (72·5)	0·001
Serum creatinine (mmol/L)	103·5 (17)	97·2 (12)	0·002

*Cases and controls were frequency matched by age. CHD=coronary heart disease; BP=blood pressure; HDL=high-density lipoprotein.

Table 1: **Baseline distributions of selected variables in cases and controls**

Blood samples were obtained from non-fasting subjects between 0830 h and 1830 h.[20] Serum was separated within 30 min of venepuncture and stored at −4°C until the next day, then at −20°C. Serum tHcy was measured by investigators unaware of case-control status. A modification of an automated assay was used, based on precolumn derivatisation with monobromobimane, followed by reverse-phase high-performance liquid chromatography with fluorescence detection.[21,22] The precision (between-day coefficient of variation) of the assay is less than 5%. The stability of analytical procedures was controlled by inserting quality control samples for every 18th sample. Replicate measurements were routinely done when the results indicated possible analysis errors. These included samples with high (≥40 μmol/L) or low (≤40 μmol/L) serum tHcy, or an unexpected relation between tHcy and the concentration of two additional aminothiols measured in the same samples, cysteine and cysteineglycine. Analyses were carried out in the Department of Clinical Biology, University of Bergen, on samples that had been stored at −20°C for 14–16 years. The methods of analysis for serum lipids and packed-cell volume have been described.[20,23,24]

We compared mean values and proportions of various cardiovascular risk factors between cases and controls by unpaired t tests, χ^2 tests, and Fisher's exact test as appropriate. Since tHcy concentrations were not normally distributed, log transformation and geometric means were used. Associations between tHcy and several risk factors for stroke were assessed in the control group by means of Spearman rank correlation coefficients. The risk (odds ratio) of stroke was examined by quarters of the ranked serum tHcy distribution relative to the first quarter. Logistic regression was used to obtain odds ratios with 95% CI adjusted for confounding factors. Age was entered as a categorical variable (four 5-year age-bands) in a logistic regression model. Body-mass index, systolic blood pressure, serum creatinine, and FEV_1 were fitted as continuous variables in the model. Social class and cigarette smoking were fitted

	r_s	p
Age	0·15	0·1
Body-mass index	−0·17	0·07
Systolic blood pressure	−0·006	0·9
Diastolic blood pressure	0·09	0·3
FEV_1	−0·08	0·3
Total cholesterol	0·15	0·1
HDL-cholesterol	0·07	0·4
Packed-cell volume	0·17	0·08
Serum creatinine	0·34	0·001

Table 2: **Spearman correlation coefficients (r_s) between serum tHcy concentration and cardiovascular disease risk factors in control subjects**

	Odds ratio (95% CI)*		
	A	B	C
tHcy (μmol/L)			
<10·3	1·0	1·0	1·0
10·3–12·49	1·2 (0·5–2·8)	1·3 (0·4–4·6)	1·2 (0·3–4·2)
12·5–15·39	2·7 (1·1–6·6)	3·3 (0·9–11·5)	2·6 (0·7–9·3)
≥15·4	4·1 (1·6–10·5)	7·4 (1·9–29·0)	4·7 (1·1–20·0)
p for trend	0·003	0·003	0·03

A=adjusted for age-group and town; B=adjusted for factors in A plus social class, body-mass index, hypertensive status, history of diabetes, cigarette smoking, FEV_1, alcohol consumption, HDL-cholesterol, and packed-cell volume; C=adjusted for factors in B plus serum creatinine concentration. *Based on data from 201 men (97 cases, 104 controls) with data for all covariates.

Table 3: **Adjusted relative risk of stroke in each quarter of tHcy distribution relative to first**

as dichotomous variables (manual/non-manual; current smoker/non-smoker), and alcohol was fitted as three variables (four categories). We adjusted for possible town effects by fitting 16 dummy variables for the 17 towns in the study.

Possible interactions between tHcy and established risk factors for stroke (age-group, hypertensive status, and smoking status) were explored in stratified analyses and by comparison of the goodness-of-fit of logistic regression models, with and without interaction terms, by the likelihood ratio test.

Results

Concentrations of tHcy were significantly higher in cases than in controls (geometric mean 13·7 [95% CI 12·7–14·8] *vs* 11·9 [11·3–12·6] μmol/L; p=0·004). Within the group of cases, there was a graded increase in the relative risk of stroke in the second, third, and fourth quarters of the tHcy distribution (odds ratios 1·3, 1·9, 2·8; trend p=0·005) relative to the first quarter. Blood pressure was substantially higher among cases than controls and a significantly higher proportion of cases than controls were manual workers, cigarette smokers, and heavy drinkers (table 1). Body-mass index, packed-cell volume, FEV_1, and serum creatinine were higher and high-density-lipoprotein (HDL) cholesterol concentrations were significantly lower in cases than in controls (table 1).

Associations between tHcy and established stroke risk factors were examined in the control group to detect possible confounding factors. There was in this group an inverse association between serum tHcy and body-mass index and a positive association with packed-cell volume both of borderline significance (table 2). A significant positive association was observed between serum tHcy and creatinine concentrations. No significant associations were seen with other continuous risk factors. Within the control group, the mean tHcy concentration was higher for the six men with pre-existing coronary heart disease at baseline than for men (n=112) without evidence of coronary heart disease at baseline (14·3 [10·5–19·6] *vs* 11·8 [11·2–12·4] μmol/L; p=0·1). tHcy concentrations were similar in the 40 smokers and 78 non-smokers (11·7 [10·5–13·0] *vs* 12·0 [11·3–12·8] μmol/L, p=0·6) and in 55 manual workers and 60 non-manual workers (11·8 [10·9–12·9] *vs* 11·8 [11·1–12·7] μmol/L; p=0·9). Similarly, no trend was observed with alcohol consumption in the control group.

Table 3 shows the effect of adjustment for potential confounders on the odds ratio for stroke in each quarter of the tHcy distribution relative to the first. The unadjusted odds ratios were essentially unchanged on adjustment for age-group and town. Further adjustment for a range of lifestyle and biological factors associated with increased risk of stroke in univariate analysis did not attenuate the association. Because higher serum creatinine concentrations were associated both with higher tHcy and with increased risk of stroke in univariate analysis, the effect of adding this variable to the logistic regression model is shown separately (table 3). The association between tHcy concentration and stroke remained significant after adjustment for serum creatinine. We did not adjust for pre-existing coronary heart disease at baseline because it is likely to be on any causal pathway between raised tHcy concentrations and stroke. However, among men without evidence of coronary heart disease at baseline (67 cases, 112 controls), the risk of stroke was significantly increased in the fourth quarter of tHcy distribution relative to the first (odds ratio 2·5 [1·1–6·1]); the odds ratios in the second and third quarters were 1·6 and 1·4, respectively.

A tHcy concentration in the fourth relative to the first quarter of the distribution was associated with a higher risk of stroke in hypertensive subjects (3·7 [1·0–13·1]) than in normotensive subjects (1·8 [0·6–5·5]), though the difference was not significant on testing for interaction (p=0·2). The association between tHcy concentration and risk of stroke for men of 50 years and older (at baseline) and for younger men were similar. There was no evidence of an interaction with cigarette smoking.

Discussion

This is the first prospective study to show a strong independent association between tHcy concentration and stroke. We observed a graded relation, without an obvious threshold, between serum tHcy concentration and risk of stroke in a representative sample of middle-aged British men. There was no evidence of attenuation of the association in multivariate analysis with adjustment for social class and for major stroke risk factors. A similar positive association between tHcy concentration and stroke was observed in men who had no evidence of pre-existing coronary heart disease at baseline. Confirming previous observations,[25] we found a significant positive association between serum tHcy and creatinine concentrations in the control group, and we showed that the association between serum tHcy and stroke was independent of serum creatinine.

These findings are consistent with the hypothesis of a causal link between raised circulating tHcy concentrations and stroke. It may be argued that tHcy is simply a marker for dietary factors linked to stroke, which are unrelated to methionine metabolism. This explanation is unlikely given the graded association observed, the clinical evidence linking very high tHcy concentrations with vascular disease, the consistent findings from cross-sectional and case-control studies in diverse settings,[2] and the biological plausibility of this hypothesis.[9] Though some earlier studies of the relation between Hcy concentrations and atherosclerotic vascular disease have used methionine loading tests,[2,26] it is now clear that measurement of tHcy in plasma or serum provides equally valid and reliable data.[27] In the context of this study, increased variability and measurement error due to the use of non-fasting samples[28] and the long period of storage[21,29] will tend to dilute associations towards the null effect. Hence, it is likely that the size of the association has been underestimated.

Our findings in this study do not accord with those from previous prospective studies of tHcy and stroke—the Physicians' Health Study cohort[12] and a Finnish

population-based study.[13] tHcy concentrations in USA physicians[12] are likely to be lower than those in the general USA population, because of higher intake of the relevant dietary cofactors. The low tHcy concentrations in the Finnish population[13] may reflect the low frequency of genes predisposing to hyperhomocysteinaemia in this population. Hence, both of those studies may have lacked power to detect significant effects of Hcy on the risk of stroke. By contrast, in a cross-sectional study (Atherosclerosis Risk in Communities study[5]) there was a strong, independent association between fasting plasma tHcy concentrations and carotid-artery intimal-medial wall thickening in symptom-free individuals. There is less potential for bias in the latter study design than in cross-sectional or conventional case-control studies with symptomatic disease as the endpoint. Similarly, Selhub and colleagues,[6] in a cross-sectional study, reported a significant association between plasma tHcy and the prevalence of significant extracranial carotid artery stenosis in men and a similar, though weaker, association in women. In that study, as in ours, the association between tHcy and cerebrovascular disease was linear, with increased risk of disease at tHcy concentrations regarded as within the normal range.

Because of the small number of events, our study has limited power to assess interactions between tHcy and other risk factors for stroke. In the Atherosclerosis Risk in Communities study,[5] the association between tHcy concentration and carotid-artery intimal-medial wall thickening was stronger in hypertensive than in normotensive subjects. In our study, the odds ratio of stroke associated with a tHcy concentration in the fourth relative to the first quarter of the distribution was twice as high in hypertensive as in normotensive subjects, though the difference was not statistically significant. From the proposed mechanisms linking high Hcy concentrations with cardiovascular disease,[9] in particular the association with vascular endothelial dysfunction,[7] a synergistic interaction with blood pressure is plausible and should be investigated in future studies.

Our findings are consistent with the hypothesis that high blood concentrations of Hcy are associated with increased risk of cerebrovascular disease. The association is strong, independent of established cardiovascular disease risk factors, and continuous across the tHcy distribution. There is a clear analogy with the relation between blood pressure and stroke. If the association is causal, the population attributable risk of stroke due to high concentrations of Hcy will be high. There is evidence that folate supplementation lowers moderately raised Hcy.[30] Our findings strengthen the case for randomised controlled trials to assess the effectiveness of interventions that reduce Hcy concentration in the prevention of stroke. Such trials will provide critical evidence regarding the reversibility or otherwise of the association between Hcy and stroke.

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