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Homocysteine and coronary heart disease risk in the PRIME study

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Abstract

Introduction: Despite recent meta-analyses suggesting that homocysteine is an independent predictor of coronary heart disease (CHD), there is debate regarding whether elevated homocysteine may be deleterious only in the presence of other risk factors, with which it acts synergistically to exert a multiplicative effect on CHD risk, emerging only as a CHD predictor in patients with pre-existing risk factors. The Prospective Epidemiological Study of Myocardial Infarction (PRIME) Study is a multicentre prospective study of 10 593 men from France and Northern Ireland, investigating cardiovascular risk factors. We investigated: (1) whether higher homocysteine is associated with increased CHD risk in the PRIME case-control cohort; (2) whether homocysteine interacts synergistically with pre-existing CHD risk factors.

Methods: Homocysteine was measured in 323 participants who had developed CHD at 5-year follow-up and in 638 matched controls.

Results: There was no significant difference in homocysteine between cases and controls (p = 0.18). Homocysteine was significantly higher in current smokers (geometric mean H9262 mol/l (interquartile range H9262 mol/l) 9.45 (7.43, 11.75)) compared with non-smokers (8.90 (7.32, 10.70); p = 0.007). There was a significant interaction between homocysteine, smoking and CHD risk (χ² = 10.29, d.f. = 2, p = 0.006).

Conclusions: These findings suggest that elevated homocysteine is significantly associated with CHD risk in current smokers.

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Keywords: Homocysteine; Coronary heart disease; Prospective cohort; PRIME; Smoking

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1. Introduction

CHD is responsible for 50% of all mortality in developed countries [1]. Classic risk factors such as smoking, male gender, hypertension and hyperlipidaemia do not fully explain the risk of developing CHD [2]. There is a large difference in incidence of CHD between Northern Ireland and France that is only partly explained by classical risk factors [3], indicating that novel risk factors are likely to play a role in CHD aetiology [4]. The PRIME study (Prospective Epidemiological Study of Myocardial Infarction) was set up to investigate possible reasons for the difference in CHD incidence between Northern Ireland and France. There are four centres: three in France (Lille in the north, Strasbourg in the East and Toulouse in the southwest) and one in Belfast, Northern Ireland.

Homocysteine is a sulphur-containing, non-proteinogenic, amino acid derived from the metabolism of the dietary protein methionine [5]. The normal concentration of total homocysteine in fasting subjects is between 5 and 15 μmol/l [6]. Most retrospective case-control studies support the hypothesis that homocysteine is a risk factor for CHD [7] which has been confirmed by meta-analyses [8–11]. However it has been suggested that hyperhomocysteinaemia is not a primary risk factor for CHD, but instead acts synergistically with pre-existing CHD risk factors to increase the likelihood of clinical CHD in subjects with atherosclerotic disease [7], thus only emerging as a CHD risk factor in populations with pre-existing disease and/or CHD risk factors.

The aim of this study was firstly to investigate prospectively whether there was an independent association between elevated homocysteine concentration and CHD risk in healthy middle-aged men; secondly, to determine whether homocysteine interacts with pre-existing conventional CHD risk factors, e.g. smoking, hypertension and hypercholesterolaemia, to exert a multiplicative effect on CHD risk.

2. Methods

Recruitment and examination methods have been described previously [3]. Participation was voluntary and all subjects signed a consent form.

2.1. Population recruitment

A total of 10593 men aged 50-59 years were recruited from industry, various employment groups and general practice between 1991 and 1993. The sample was recruited to match broadly the social class structure of the background population.

2.2. Personal history

Each subject completed self-administered questionnaires on demographic, socio-economic factors and dietary habits. Responses were checked by medical staff, and additional data were collected on tobacco and alcohol consumption and physical activity during clinic attendance.

The London School of Hygiene Cardiovascular (Rose) Questionnaire for Chest Pain on Effort and Possible Infarction [12] was also administered.

2.3. Clinical examination

Baseline investigations included a standard 12-lead electrocardiogram, and standardised blood pressure measurements using an automatic sphygmomanometer (Spen- gler SP9). Anthropometric measurements included height, weight, and waist and hip circumferences. From these measurements, body mass index (BMI) and waist-hip ratio (WHR) were calculated.

2.4. Pre-existing CHD

Subjects were considered to have CHD at entry if they reported at least one of the following events:

(i) Myocardial infarction (MI) and/or angina pectoris diagnosed by a physician.

(ii) Electrocardiographic evidence of MI.

(iii) A positive answer to the Rose questionnaire.

There were 9758 subjects without evidence of CHD at study entry, 7359 in France and 2399 in Northern Ireland.

2.5. Case-control selection and follow-up

Subjects were contacted annually by letter and asked to complete a clinical event questionnaire. Coronary events were defined as the presence of at least one of the following: non-fatal MI, death from CHD or angina pectoris [13]; these were independently validated by a Medical Committee, comprised of one member from each PRIME centre and three independent cardiologists. Medical information from proposed cases was reviewed by the Committee and assigned a code according to a strict protocol.

Five-year follow-up has been completed. The total number of new CHD cases identified was 335. Each case was matched to two controls who were study participants of the same age (±3 years), recruited in the same centre on the same day as the corresponding case (±2 days) and were free of CHD on the date of the ischaemic event of the case, giving 670 controls.

2.6. Laboratory measurements

Venous blood samples were collected after a 12-h fast and centrifuged within 4 h. All measurements were performed blind to the investigating centre and samples from all four centres were included in each analytical run.

Measurement of total and HDL cholesterol, triglycerides, ApoB, glutathione peroxidase (GPx), fibrinogen, apoA1 and Lp(a) were performed at the start of the study on all subjects. Glucose, bilirubin, cystatin C, B12, folate and homocysteine
(tHcy) were only measured on subjects involved in the case-control study.

Plasma for lipid analysis was sent weekly at 4°C to SERLIA-INSERM U325 (Pasteur Institute, Lille, France), the coordinating laboratory. Methods used to measure baseline lipid parameters, GPx and fibrinogen have been described previously [3].

Bilirubin and glucose was measured using automated colorimetric assays (Sigma Diagnostics, UK; Randox, UK). Homocysteine was measured by HPLC with fluorescence detection according to the method of Ubbink and colleagues [14]. Serum folate and Vitamin B12 were measured simultaneously by radioassay (ICN Pharmaceuticals, USA). Cystatin C was measured by immunonephelometry (Dade Behring, Reuil-Malmaison, France).

2.7. Statistical analysis

Variables with a skewed distribution (bilirubin, glucose, fibrinogen, ApoB, folate, B12, Lp(a), homocysteine and triglycerides) were logarithmically transformed and results summarised as geometric mean and interquartile range. Pearson correlation coefficients were used to investigate the associations between homocysteine concentration and other CHD risk factors separately in cases and controls.

One-way ANOVA and independent samples t-tests were used to examine the associations between homocysteine concentration and centre, smoking and alcohol intake separately in cases and controls. Comparisons between cases and controls for continuous variables were made using ANOVA, adjusting for case-control triplets by the inclusion of terms representing triplets in the model; comparisons were summarised as a difference in means between cases and controls, adjusted for triplets; p-values are given. For variables requiring log transformation the data were summarised as a ratio of geometric means in cases and controls, adjusted for triplets. Conditional logistic regression was used to examine the relationships between smoking, alcohol intake and physical activity and case-control status.

To assess the relationship between homocysteine concentration and CHD risk, the subjects were divided into fifths by quintiles of the distribution of homocysteine concentration in the control group. Conditional logistic regression was performed to estimate the odds of CHD risk for each fifth of the homocysteine distribution relative to the lowest fifth. Tests for heterogeneity in CHD risk between the five categories are presented. Tests for trend in CHD risk across the five categories were obtained by fitting linear terms in the conditional logistic model. Tests were also performed to check for non-linearity in the relationship between risk and the fifths of homocysteine concentration. Two further conditional logistic models were then fitted. In the first model, the odds ratios for homocysteine concentration were adjusted for classical risk factors, namely WHR, diastolic blood pressure (DBP), smoking, total cholesterol, HDL and triglycerides. In the second model, the odds ratios were adjusted for the classic risk factors but also factors linked with homocysteine: namely B12, folate and cystatin C. Statistical significance was assessed using Wald’s test or by comparing the fits of the nested models using likelihood-ratio tests. To test for differences between countries in the relationship between homocysteine and CHD, interaction terms were added in the conditional logistic regression model.

In addition, the results were tested for interaction between homocysteine concentration and smoking category regarding CHD risk. Within each smoking category, a test was performed to determine whether the differences in risk between fifths of homocysteine deviated from a linear relationship. This was done by comparing the fits of a model specifying a linear relationship with one permitting any form of relationship. These tests were then pooled over the three smoking categories to give a composite test for deviation from linearity in the relationship between homocysteine and CHD risk.

In order to justify the assumption of linearity in the trend within each smoking sub-group, the fit of a model specifying linear relationships was compared with one permitting any form of relationship to give a likelihood-ratio test for non-linearity. Statistical significance was assessed as p < 0.05. All odds ratios are quoted with 95% confidence intervals. Analyses were performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA) and STATA 8.0 statistical software (Statcorp, College Station, TX, USA).

3. Results

Correlations of variables with homocysteine concentration are shown in Table 1. In controls, homocysteine was found to have a weak positive correlation with WHR and a weak inverse relationship with Lp(a). In cases, it was positively correlated with both DBP and fibrinogen, and in both cases and controls there was a strong positive correlation with cystatin C (p < 0.001 for both cases and controls) and a strong negative correlation with folate (p < 0.001 for both cases and controls).

Homocysteine was significantly higher and B12 significantly lower in Belfast compared with France (Table 2, p < 0.001 for both), although there was no significant difference in folate between countries. Because controls are matched to cases for centre of examination as well as for age and date of examination, this difference in homocysteine between Belfast and France did not have to be considered in further analysis.

3.1. Smoking

Homocysteine was significantly higher in current smokers compared with non-smokers (never and ex-smoker categories combined) (geometric mean, μmol/l (IQ range, μmol/l); current smokers 9.45 (7.43, 11.75); non-smokers 8.90 (7.32, 10.70); p = 0.007), although the difference remained signif-
Table 1
Pearson correlation coefficients for the association between plasma tHcy concentration and other cardiovascular risk factors for cases and controls (cases: controls: 638; 323)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of subjects, controls: cases</th>
<th>Correlation coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Age</td>
<td>638; 323</td>
<td>-0.011</td>
</tr>
<tr>
<td>BMI</td>
<td>638; 323</td>
<td>-0.009</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>636; 322</td>
<td>0.008*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>637; 322</td>
<td>0.054</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>637; 322</td>
<td>0.021</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>634; 321</td>
<td>0.054</td>
</tr>
<tr>
<td>Triglycerides†</td>
<td>634; 321</td>
<td>0.061</td>
</tr>
<tr>
<td>Glucose§</td>
<td>577; 302</td>
<td>-0.028</td>
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<tr>
<td>Fibrinogen§</td>
<td>623; 311</td>
<td>0.070</td>
</tr>
<tr>
<td>ApoB§</td>
<td>634; 321</td>
<td>0.076</td>
</tr>
<tr>
<td>Folate§</td>
<td>596; 306</td>
<td>-0.143†</td>
</tr>
<tr>
<td>B12§</td>
<td>596; 306</td>
<td>-0.058</td>
</tr>
<tr>
<td>Lp(a)§</td>
<td>632; 321</td>
<td>-0.084*</td>
</tr>
<tr>
<td>GPx§</td>
<td>581; 301</td>
<td>-0.087</td>
</tr>
<tr>
<td>Bilirubin§</td>
<td>414; 209</td>
<td>-0.008</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>635; 320</td>
<td>0.195†</td>
</tr>
<tr>
<td>ApoA-1</td>
<td>634; 321</td>
<td>-0.013</td>
</tr>
</tbody>
</table>

Significance indicated by *p<0.05; †p<0.01; ‡p<0.001. §Denotes variables which have been log transformed.

...
Comparison of baseline risk factors between cases and controls by analysis of variance using logarithmically transformed data with inclusion of terms for case-control triplet in the model

Table 3

Comparison of baseline risk factors between cases and controls by analysis of variance with inclusion of terms for triplet in the model

Variable | Number of cases, controls | Cases, mean (S.D.) | Controls, mean (S.D.) | Adjusted difference between means (95% CI) | p-Value
--- | --- | --- | --- | --- | ---
BMI (kg/m²) | 335, 670 | 23.0 (4.3) | 23.0 (3.2) | 0.64 (0.18, 1.10) | 0.007
Waist-to-hip ratio | 334, 669 | 0.87 (0.06) | 0.87 (0.035) | 0.013 (0.006, 0.020) | <0.001
Systolic blood pressure (mmHg) | 334, 669 | 140.0 (21.8) | 133.8 (18.3) | 6.2 (3.7, 8.7) | <0.001
Diastolic blood pressure (mmHg) | 334, 669 | 86.5 (12.2) | 83.5 (11.9) | 3.0 (1.5, 4.5) | <0.001
Total cholesterol (mmol/l) | 333, 666 | 6.03 (1.0) | 5.78 (1.0) | 0.25 (0.12, 0.39) | <0.001
HDL cholesterol (mmol/l) | 333, 666 | 1.14 (0.33) | 1.22 (0.33) | -0.08 (~0.12, -0.04) | <0.001
Cystatin C (mg/l) | 332, 665 | 0.81 (0.15) | 0.78 (0.14) | 0.03 (0.01, 0.05) | <0.001

The data are presented as crude means (standard deviation (S.D.)) in cases and controls and then as the difference (case minus control) adjusted for the case-control triplet. The p-value is quoted for a comparison of the adjusted differences; n = maximum number.

Table 4

Comparison of baseline risk factors between cases and controls by analysis of variance using logarithmically transformed data with inclusion of terms in the model

Variable | Number of cases, controls | Cases, geometric mean (IQ range) | Controls, geometric mean (IQ range) | Adjusted ratio of geometric means (95% CI) | p-Value
--- | --- | --- | --- | --- | ---
Bilirubin (µmol/l) | 216, 434 | 7.95 (5.32, 12.33) | 9.07 (6.16, 12.76) | 0.87 (0.79, 0.98) | 0.005
Glucose (mg/dl) | 312, 620 | 7.34 (5.19, 10.81) | 7.25 (5.09, 10.67) | 1.00 (0.93, 1.07) | 0.95
Triglycerides (mmol/l) | 323, 654 | 3.47 (2.90, 4.15) | 3.25 (2.75, 3.72) | 1.07 (1.03, 1.10) | <0.001
Fibrinogen (g/l) | 356, 666 | 3.18 (1.19, 5.17) | 2.17 (1.08, 4.48) | 1.09 (1.05, 1.12) | <0.001
Homocysteine (µmol/l) | 323, 638 | 9.07 (7.26, 10.91) | 8.97 (7.26, 10.91) | 1.00 (0.99, 1.01) | 0.93
Cystatin C (mg/l) | 316, 613 | 0.81 (0.15) | 0.78 (0.14) | 0.03 (0.01, 0.05) | <0.001

The data are presented as crude geometric means (interquartile range (IQ)) in cases and controls and then as the ratio (case/control) adjusted for the case-control triplet. The p-value is quoted for a comparison of the adjusted means.

Table 5

Conditional logistic regression analysis showing the unadjusted and adjusted odds ratio (OR) of CHD risk for fifths of homocysteine concentration based on control values

| Homocysteine (µmol/l) | Unadjusted OR (95% CI), number of subjects 943 | p-Value | Adjusted model 1 OR (95% CI), number of subjects 931 | p-Value | Adjusted model 2 OR (95% CI), number of subjects 821 | p-Value
--- | --- | --- | --- | --- | --- | ---
<7.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00
7.01–8.17 | 1.06 (0.67, 1.66) | 0.84 | 1.09 (0.68, 1.75) | 0.72 | 0.93 (0.65, 1.37) | 0.78
8.18–9.63 | 1.05 (0.68, 1.62) | 0.82 | 1.06 (0.67, 1.67) | 0.81 | 1.06 (0.64, 1.75) | 0.82
9.64–11.05 | 1.05 (0.68, 1.63) | 0.82 | 1.05 (0.69, 1.51) | 0.81 | 0.88 (0.52, 1.49) | 0.63
>11.41 | 1.15 (0.75, 1.78) | 0.53 | 1.10 (0.69, 1.75) | 0.69 | 1.01 (0.60, 1.49) | 0.98

Unadjusted model test for heterogeneity: $\chi^2 = 11.90$, d.f. = 9, $p = 0.22$. Test for trend $\chi^2 = 10.29$, d.f. = 2, $p = 0.006$. A test for departures from linearity pooled across the three smoking categories was non-significant ($\chi^2 = 11.90$, d.f. = 9, $p = 0.22$).

Table 6

Relationship between homocysteine concentration and CHD risk according to smoking status, adjusted for WHR, DBP, total cholesterol, HDL cholesterol, triglycerides, glucose, alcohol, physical activity, ApoB, fibrinogen, GPs, B₆, folate, Lp(a) and cystatin C

| Smoking category | Odds ratio (95% CI) | p-Value |
--- | --- | ---
Never-smoker | 0.76 (0.57, 1.02) | 0.07 |
Current smoker | 1.50 (1.05, 1.60) | 0.015 |
Ex-smoker | 0.92 (0.75, 1.12) | 0.40 |

The odds ratios represent the increase in risk per step up the 5-point homocysteine scale. Test for differences in trend $\chi^2 = 10.29$, d.f. = 2, $p = 0.006$. Test for non-linearity $\chi^2 = 11.90$, d.f. = 9, $p = 0.22$.
increase in CHD risk per step up the 5-point homocysteine scale.

There was no interaction between homocysteine and blood pressure or total cholesterol, and therefore homocysteine was not found to modify the relationship between these factors and CHD risk (data not shown).

4. Discussion

4.1. Homocysteine, CHD risk and interaction with smoking

In the PRIME study there was no significant difference in homocysteine between cases and controls and no association between homocysteine and CHD risk. Studies have not consistently shown that homocysteine is a risk factor for CHD. Although recent meta-analyses [8,9,11] and most retrospective case-control studies support the hypothesis that homocysteine is a CHD risk factor [7], several prospective studies have failed to demonstrate an association between homocysteine concentration and CHD risk [15–19].

It has been suggested that hyperhomocysteinaemia may not be a primary risk factor for CVD, but may require pre-existing CHD risk factors with which to interact to increase CHD risk synergistically, thereby only increasing the likelihood that pre-existing atherosclerotic disease will progress to clinical disease [15]. In the PRIME case-control cohort, such an interaction was observed between homocysteine and smoking status. The relationship between homocysteine and CHD risk was significantly different in never, current and ex-smokers. A significant increase in CHD risk with increasing homocysteine was found only in current smokers indicating that homocysteine and smoking interact to increase CHD risk.

This result agrees with the findings of the European Concerted Action Project [20]. In that study, an independent dose-response between homocysteine and CHD risk was detected. Additionally, interaction effects were noted, with elevated homocysteine causing a multiplicative effect on risk with smoking, hypertension and hypercholesterolaemia, increasing it 2–3-fold. There was a significant interaction between smoking and homocysteine (p = 0.04) with the odds ratios for vascular disease in separate smoking categories found to be independent of B-vitamin and folate status [21].

There are a number of plausible reasons why smoking and hyperhomocysteinaemia interact to have a multiplicative effect on risk. These include their similar effects on the vascular system or increasing oxidative stress [21].

4.2. Homocysteine, folate and smoking

In the PRIME case-control cohort, homocysteine was significantly higher, while folate was significantly lower, in current smokers compared with non-smokers (cases and controls combined) with no significant difference in B12 status. A number of recent studies have shown smokers to have reduced folate levels [22–26], with one study suggesting a direct and independent effect on erythrocyte folate concentration [25].

However, because smokers have been shown to have a lower vitamin intake than non-smokers [27,28] without nutritional information it is impossible to state whether smoking has an independent effect on folate concentration in PRIME. The absence of a relationship between B12 concentration and smoking is in keeping with most previous findings [24–26,29], although not all [30].

Given the effect of smoking on folate, it is not surprising that smoking has been associated with homocysteine concentration. As in PRIME, previous studies have demonstrated significantly higher homocysteine in current smokers compared with non-smokers [30–32]. Furthermore, in PRIME homocysteine concentration was significantly correlated with the quantity smoked, suggesting a dose-dependent effect on homocysteine concentration, also shown in previous studies [26,31,32].

The mechanism by which smoking raises homocysteine levels is not known. Constituents of cigarette smoke may act upon the enzymes and cofactors involved in the remethylation of homocysteine to methionine, e.g. tetrahydrofolates [33,34], and methionine synthase [35]. Alternatively, increased total homocysteine could be due to elevated reduced-homocysteine in smokers, possibly as a defence mechanism against oxidative stress [36].

4.3. Differences by country

Homocysteine concentration was significantly higher and B12 significantly lower in Northern Ireland compared with France, with no significant difference in folate. This suggests that the difference in homocysteine between countries may be due to differences in B12 status, although it is possible (but unlikely) that genetic differences between the populations may also be contributory.

4.4. Homocysteine, folate and B12

In the PRIME case-control cohort, there was a significant negative correlation between homocysteine concentration and plasma folate concentration in both cases and controls. This is as expected, because the folate cycle is linked to the homocysteine remethylation pathway. MTHFR reduces 5,10-methylene THF to 5-methyl THF, which is the only reaction in the cell generating 5-methyl THF. Methionine synthase reduces 5-methyl THF to methionine, which acts upon the enzymes and cofactors involved in the remethylation of homocysteine to methionine. Therefore, low plasma folate reduces homocysteine remethylation.

However, there was no correlation between B12 and homocysteine concentration in the PRIME study. It is possible that B12 may only correlate with homocysteine concentration in the presence of a B12 deficiency, when it then becomes the rate limiting factor in the remethylation of homocysteine to methionine. The normal values are, for B12 greater
than 160 pg/ml and for folate, greater than 1.5 ng/ml. B12 was measured in 900 subjects in the PRIME cohort. Only 15 of these had a B12 level less than 160 pg/ml. Therefore, few subjects in the PRIME cohort were deficient in B12, and this may account for the lack of association with homocysteine. Other studies have also failed to demonstrate an association between B12 and homocysteine concentration [37–40].

4.5. Homocysteine and cystatin C

Homocysteine concentration is dependent on renal function, although few studies have controlled for renal function. Cystatin C is a more sensitive marker than creatinine [41], thus the highly significant positive correlation between homocysteine and cystatin C in PRIME is as expected. It is interesting that this association is so marked in subjects with essentially normal renal function.

5. Conclusion

The results of the PRIME study suggest that in healthy, middle-aged male populations homocysteine is not an independent risk factor for CHD. However in PRIME elevated homocysteine did act as a CHD risk factor in current smokers. This suggests that homocysteine requires an interaction with another CHD risk factor before playing a role in elevating CHD risk. Thus, future research may benefit by focusing on the role of homocysteine in CHD in patients with pre-existing disease and/or risk factors.

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