ELSEVIER

Atherosclerosis 154 (2001) 659–666

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

The effect of the C677T and A1298C polymorphisms in the methylenetetrahydrofolate reductase gene on homocysteine levels in elderly men and women from the British regional heart study

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Received 26 October 1999; received in revised form 17 April 2000; accepted 28 April 2000

Abstract

Total blood levels of homocysteine (tHcy) have been shown to depend on both environmental and genetic factors, and to be associated with the risk of developing atherosclerosis with its complications of coronary heart disease (CHD) and stroke. In this study, 408 men and 346 women from two towns, Dewsbury and Maidstone were examined for tHcy levels and genotyped for the C677T and the A1298C polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene. Blood tHcy was significantly higher in men from the CHD high risk town of Dewsbury (12.7 μmol/l) than in the low CHD risk town of Maidstone (11.5 μmol/l) $P < 0.001$, but not in women (10.7 vs. 10.5 μmol/l), with women in both towns, thus, showing significantly lower tHcy than men. There was no difference between towns in folate or vitamin B_{12} levels but the conventional inverse relationship with tHcy was seen. Smoking men and women from both towns had significantly higher tHcy and lower folate levels than non-smoking individuals ($P < 0.001$). The frequency of the 677T allele in Dewsbury was 0.35 (95% CI; 0.32–0.39) compared with 0.29 (95% CI; 0.26–0.32) in Maidstone ($P < 0.01$). Similar frequency difference of borderline statistical significance was seen both for men ($P = 0.054$) and women ($P = 0.048$) in both the towns, suggesting a true regional frequency difference. The effect of the 677T on tHcy was highly significant in the group as a whole with the most profound effect seen in men (12.0 μmol/l for CC vs. 14.1 μmol/l for TT, $P < 0.001$). By contrast, there was no significant effect of the A1298C polymorphism on tHcy, folate or vitamin B_{12} levels, with no evidence for an interaction with the C677T genotype. The regional differences in tHcy levels were still present after the adjustment for folate and vitamin B_{12} levels, smoking and the effect of the C677T polymorphism. This suggests that there may be other unidentified factors, either environmental or genetic, affecting tHcy levels, and thus potentially having an impact on the risk of developing hyperhomocysteinaemia and CHD. These observations may have a bearing on regional differences in tHcy levels and the variation in CHD risk between regions in the UK.

Keywords: Homocysteine; Methylene tetrahydrofolate reductase polymorphisms; British regional heart society; Coronary heart diseases

1. Introduction

Epidemiological studies have identified a number of risk factors that contribute to the development of atherosclerosis and coronary heart disease (CHD). One independent risk factor identified for CHD is raised plasma homocysteine concentration [1,2]. A relationship between total blood levels of homocysteine (tHcy) and CHD has been demonstrated in the UK [3] and mild elevation of tHcy is frequently seen in the general population [4].

There is a well documented inverse relationship between tHcy level, folate and vitamin B_{12} levels [5], and homocysteine metabolism depends on vitamins such as

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0021-9150/01/$ - see front matter
PII: S0021-9150(00)00522-0

folate, cobalamin and pyridoxal-5-phosphate that act as co-substrates or co-factors during the remethylation or transulphuration pathways [6,7]. A number of enzymes are involved in these two interconnected pathways, and methylene-tetrahydrofolate reductase (MTHFR), cystathionine beta synthase (CBS), are those most extensively studied [6,7]. Variation in these genes may, thus, be important in determining tHcy levels in the general population.

A common missense mutation, C677T has been identified in the MTHFR gene where alanine (A) is substituted by valine (V), which results in a thermolabile variant of the reductase [8,9]. This thermolabile variant has been consistently associated with mild elevations in tHcy levels [10]. The relative frequency of the coding allele for valine is about 35% in most populations, although this figure varies substantially between different populations [11]. The recessive nature of the effect of this amino acid substitution in determining tHcy levels has been confirmed by a number of studies [10]. There is, however, a conflict in the literature regarding whether this mutation is an independent risk factor for elevated levels of tHcy and cardiovascular disease (reviewed in [10]).

Another polymorphism in the MTHFR gene has been reported, A1298C which results in a substitution of glutamate for an alanine residue [12]. The A1298C mutation has been associated with risk of neural tube defects and shown to have reduced enzymatic activity [12], but there is no reported association with levels of tHcy in healthy subjects.

In this study, we examined the effect of the C677T and A1298C polymorphisms in the MTHFR gene in healthy elderly men and women from Dewsbury and Maidstone. These two towns were selected among the 24 towns participating in the BRHS because they have an approximately two-fold difference in coronary heart disease risk [13].

2. Materials and methods

2.1. Subjects

The British Regional Heart Study (BRHS) is a prospective study of cardiovascular disease in 7735 men aged 40–59 years who have been followed up since entry to the study in 1978–1980. Men were selected from the age–sex register of a single General Practice in each of 24 British towns. In 1996, surviving men in two study towns, Dewsbury in Northern England and Maidstone in Southern England, were invited for follow up [13]. A random sample of women of similar age to the men (56–77 years) were also selected from the age–sex register of the general practices used in the original survey. A self-administered questionnaire was used to obtain information on lifestyle factors and pre-existing disease. Complete measurements were made in 408 men and 346 women (response rates 83 and 69%, respectively).

2.2. Blood collection and measurements

Non-fasting venous blood samples were obtained at the baseline examination between 8:30 and 18:30 h. Serum was separated on site within 1 h of venepuncture, stored at 4°C until the following day and at −20°C thereafter. Serum homocysteine was determined, blind to clinical status, in the Department of Pharmacology, University of Bergen. A modification of an automated assay was used, based on pre-column derivatization with monobromobimane, followed by reverse phase HPLC with fluorescence detection [14]. The between-day coefficient of variation is less than 5%. Replicate measurements were routinely performed, all values presented are based on the mean of two values. Serum folate and vitamin B_{12} levels were measured at the Department of Clinical Pathology Whittington Hospital London, using the Bayer Immuno 1 analyser. The vitamin B_{12} and folate assays were performed using a standard competitive binding technique.

2.3. DNA extraction and genotyping

DNA was extracted by the salting out method as previously described [15]. The DNA was stored diluted in Beckman plates with 96-deep wells as described [16] and aliquoted into 96-well Omnigene plates and dried. The PCR reaction mixture consisted of 50 mmol/l KCL, 10 mol/l Tris (pH 8.3), 0.1 g/l gelatin (Sigma, UK) 2 mmol/l of each dNTP; 15 mmol/l $MgCl_2$; 0.02% W-1 (GIBCO, UK), 0.1 U of *Taq* polymerase (GIBCO) per 10 μl PCR and 0.120 μM of each of the primers. For the MTFHR C677T the primers used were as described by Frosst et al. [9]. The PCR conditions were 95°C for 5 min, 60°C for 1 min, and 72°C for 1 min for one cycle, and subsequently 35 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, and finally 72°C for 5 min. For the MTHFR A1298C, the primers used were as described by van der Put et al. [12]. The PCR conditions were 95°C for 5 min, 52°C for 2 min, and 72°C for 2 min for one cycle, and subsequently 35 cycles at 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. The C677T PCR product was subjected to restriction enzyme analysis by digestion with 2 U of *Hin*fI (NEB Company) restriction endonuclease per 8 μl of the PCR sample at 37°C for 1 h, in the buffer recommended by the manufacturer. The product A1298C was subjected to restriction enzyme analysis by digestion with 2 U of *Mbo*II (Helena Company) restriction endonuclease per 8 μl of the PCR sample at 37°C overnight, in the buffer recommended by the manufac-

Table 1
Characteristics of the cohort by gender

	Men ($N = 408$)[a]	Women ($N = 346$)[a]	P-value
Age[b]	66.3 (5.6)	65.7 (5.9)	0.19
tHcy (μmol/l)[c]	12.1 (6.7–21.8)	10.5 (5.7–19.3)	<0.0001
Folate (nmol/l)[c]	6.0 (2.4–15.2)	5.9 (2.4–15.8)	0.53
B_{12} (pmol/l)[c]	330 (159–685)	384 (260–821)	<0.01
Smokers (%)[d]	20.8	16.8	0.16

[a] The number of individuals from whom tHcy, vitamin B_{12} and folate measurements were obtained.
[b] Mean ± S.D.
[c] Geometric means with 95% normal range.
[d] Information on smoking was only available for 403 men and 346 women.

turer. The samples were then loaded on to an ethidium bromide prestained microplate array diagonal gel electrophoresis gel (MADGE) [16], electrophoresed for 1 h 20 min for the C677T and 45 min for the A1298C, and scored for the genotypes. Genotypes were read blind by two workers and any discrepancies were resolved by rePCR and digestion.

2.4. Statistical analysis

Statistical analyses were carried out using the SAS package. The distributions of tHcy, folate and vitamin B_{12} were positively skewed and a logarithmic transformation was used in all tests which assume normality for the dependent variable. All values in the Tables are shown as geometric means. Analysis of covariance was used to obtain age adjusted means of the logged data for each genotype group and to examine town and sex differences in tHcy levels after adjusting for the effects of MTHFR, folate, vitamin B_{12}, smoking, and age, and to examine the differences in tHcy levels between towns after adjusting for the effects of MTHFR, folate, vitamin B_{12}, smoking and age. Pearson correlation coefficients were used to examine the relationship between log tHcy and log vitamin B_{12}, log folate, and age. Linkage disequilibrium was measured as described by the method of Chakravatii et al. [17].

3. Results

3.1. Physical and biochemical characteristics of the sample

Table 1 shows the physical and biochemical characteristics of the cohort according to their gender. Men had 15% higher tHcy levels ($P < 0.0001$), and 14% lower vitamin B_{12} levels ($P < 0.0001$), than women, but the concentration of serum folate was similar in both groups. As expected, a strong negative and highly significant correlation ($P < 0.001$) between tHcy, and both folate and vitamin B_{12} was seen in both men ($r = -0.52$, $r = -0.39$) and women ($r = -0.43$, $r = -0.35$), respectively.

The difference in tHcy levels between men and women was not explained by a difference in vitamin B_{12} levels, as it was still seen after adjustment for vitamin B_{12}. The difference could not be explained in terms of folate levels, age or smoking, as the proportion of smokers was similar in both sexes. Smokers had, however, significantly higher levels of tHcy, which was about 18% ($P < 0.001$) and 11% ($P < 0.05$) higher than in non-smoking men and women, respectively (Table 2). Compared with non-smokers, both male and female smokers had 23% lower folate ($P < 0.01$ for both genders).

3.2. tHcy levels by town

As shown in Table 3, when levels of tHcy were compared between the two towns, men from Dewsbury had levels of tHcy approximately 10% higher than those from Maidstone (12.7 vs. 11.5 μmol/l, $P < 0.01$). The folate and vitamin B_{12} levels were, however, similar in both towns. No such difference in tHcy concentration was observed for women. This difference in tHcy was still seen after adjustment for the levels of vitamin B_{12} and folate, age and smoking ($P < 0.001$).

Table 2
Total serum homocysteine, serum folate and vitamin B_{12} (age adjusted geometric means, 95% CI) by smoking status and gender

Men				Women			
Smoking[a]	tHcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)	Smoking[a]	THcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)
No ($N = 318$)	11.6 (11.3–12.0)[b]	6.4 (6.1–6.7)[b]	338 (324–353)	No ($N = 284$)	10.4 (10.0–10.7)[c]	6.2 (5.8–6.5)[b]	386 (370–404)
Yes ($N = 84$)	13.7 (12.9–14.6)	4.9 (4.4–5.4)	315 (291–342)	Yes ($N = 57$)	11.5 (10.7–12.4)	4.8 (4.3–5.4)	355 (321–391)

[a] Smoking information not available for all.
[b] $P < 0.01$, between smokers and non-smokers.
[c] $P < 0.05$.

Table 3
Total serum homocysteine, serum folate and vitamin B_{12} (age adjusted geometric mean, 95% CI) by town and gender

	Men				Women		
	tHcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)		tHcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)
Dewsbury ($N=194$)	12.7 (12.2–13.2)[a]	6.2 (5.8–6.6)	326 (309–344)	Dewsbury ($N=158$)	10.7 (10.2–11.2)	6.0 (5.6–6.4)	395 (372–419)
Maidstone ($N=214$)	11.5 (11.1–12.0)	6.0 (5.6–6.4)	337 (320–354)	Maidstone ($N=188$)	10.5 (10.0–10.9)	5.9 (5.5–6.3)	372 (352–392)

[a] $P<0.01$, between towns.

Table 4
Total serum homocysteine, serum folate and vitamin B_{12} (age adjusted geometric means, 95% CI)

	Men				Women			
	N	tHcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)	*N*	tHcy (μmol/l)	Folate (nmol/l)	B_{12} (pmol/l)
(a) According to C677T genotype								
CC	184	12.0 (11.5–12.5)[a]	6.1 (5.7–6.5)[b]	330 (313–349)	161	10.2 (9.8–10.7)	6.0 (5.6–6.5)[b]	400 (376–425)
CT	188	11.9 (11.5–12.4)[a]	6.2 (5.8–6.6)[b]	330 (313–348)	149	10.7 (10.2–11.2)	6.0 (5.6–6.5)[b]	370 (348–394)
TT	36	14.1 (12.8–15.5)	5.1 (4.3–5.9)	337 (298–381)	36	11.1(10.1–12.2)	5.0 (4.3–5.9)	361 (317–411)
(b) According to A1298C genotype[c]								
AA	211	12.2 (11.7–12.7)	6.0 (5.7–6.4)	338 (322–355)	164	10.5 (10.0–11.0)	5.9 (5.5–6.4)	383 (360–406)
AC	151	12.1 (11.5–12.6)	6.1 (5.6–6.6)	320 (302–340)	158	10.6 (10.1–11.1)	5.9 (5.5–6.4)	379 (356–402)
CC	32	12.4 (11.2–13.7)	5.5 (4.6–6.4)	350 (308–397)	23	10.2 (9.0–11.5)	5.8 (4.8–7.0)	401 (341–470)

[a] $P<0.01$.
[b] $P<0.05$.
[c] Genotypes were obtained for only 394 men and 345 women.

3.3. Allelic frequencies of the C677T and A1298C polymorphisms

In the whole sample, the frequency of the T allele of the C677T polymorphism was 0.32 (95% CI; 0.30–0.34) whereas the frequency of the C allele of the A1298C polymorphism was 0.28 (95% CI; 0.26–0.31). Both polymorphisms were in Hardy–Weinberg equilibrium, and there was a significant linkage disequilibrium between them ($\Delta=0.27$, $P<0.01$). There was a statistically significant difference in the frequency of the T allele between the two towns. The frequency of the 677T allele in Dewsbury was 0.35 (95% CI; 0.32–0.39) compared with 0.29 (95% CI; 0.26–0.32) in Maidstone ($P<0.01$). This was reflected in the higher proportion of homozygotes for the 677T allele, seen in Dewsbury compared with Maidstone (12.8 vs. 6.7%, $P<0.05$) and no frequency differences between men and women in both towns. There was no such frequency difference observed for the A1298C polymorphism between towns.

3.4. Effect of the C677T and A1298C polymorphisms in the MTHFR gene on tHcy, folate and vitamin B_{12} levels

As shown in Table 4a, there was a highly significant effect of the C677T polymorphism on tHcy levels in men, with those homozygous for the thermolabile variant having higher tHcy ($P<0.001$) than either of the other two genotype groups. Folate and vitamin B_{12} levels were not, however, significantly different. In women a similar relationship between genotype and tHcy levels was observed although the differences were not statistically significant apart from folate levels, where individuals homozygous for the thermolabile variant had lower folate ($P<0.05$).

The proportion of homozygotes for the thermolabile variant was significantly higher in the highest tHcy quartile in men and women combined, (highest vs. lowest quartile 14.1 vs. 7.0%, $P=0.03$). When men and women were examined separately (Fig. 1), there was a considerable difference in the distribution of 677TT

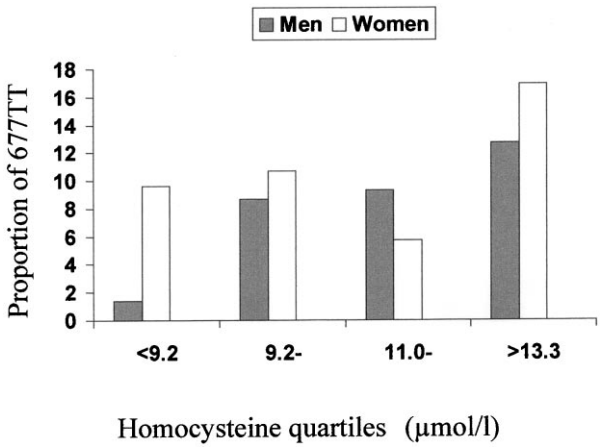

Fig. 1. The proportion of 677TT homozygotes by quartiles of tHcy, ($P = 0.02$ for men, NS for women).

between quartiles of tHcy. In men with tHcy levels in the lowest quartile, the 677TT frequency was only 1.5%, compared with 12.7% in the top quartile ($P = 0.03$), gradually rising between the quartiles. In women, however, the distribution of 677T homozygotes was not significantly different between the highest and lowest quartiles of tHcy (10.2 vs. 17.0%), with 677TT homozygotes evenly distributed between all quartiles. When compared between the towns, similar results were obtained (data not shown).

By contrast, the A1298C polymorphism was not associated with significantly different tHcy levels, folate or vitamin B_{12} levels as shown in Table 4b. It has been suggested [12] that the 677T and 1298C alleles are always in trans. This was not observed in our sample, where 25 subjects are in cis. These results were confirmed by repeat genotyping of these 25 samples. When genotypes for both mutations were combined, as shown in Table 5, individuals homozygous for both rare alleles, had the lowest tHcy levels, but not significantly lower when compared with any of the other genotypes.

3.5. Regression analysis

Regression analysis was carried out to evaluate the percentage of the variance of tHcy explained by the C677T polymorphism in the MTHFR gene, as well as nutritional and life style factors. In men, a model including age, smoking, levels of folate and vitamin B_{12} explained 35% of the variance of tHcy levels, and in women 30.7%, while the C677T polymorphism explained only an additional 2% of the variance of tHcy levels in men and 1.1% in women.

4. Discussion

The subjects examined in this study were from two of the 24 towns in the BRHS recruited from 1978–1980, namely Dewsbury and Maidstone, where subjects, still alive, were re-contacted in 1996. At the same time, women, matched for age, were recruited and included in the study. The whole sample is, thus, representative for the men and women of older adulthood. Folate, vitamin B_{12} and tHcy were measured and DNA was also extracted from these individuals.

A systematic geographic variation in tHcy levels has previously been shown in the whole of the BRHS cohort where a gradient in tHcy concentration from high to intermediate to low tHcy was seen from Scotland to Northern England to Southern England, respectively [3]. This was also seen in older British adults in the 1995–1996 National Diet and Nutrition Survey [18]. Our finding of different tHcy levels in men between the North of England, high-CHD-risk town of Dewsbury, and the South of England, low-CHD-risk town of Maidstone, confirm these findings, although the explanation for this difference is not obvious. It is, however somewhat surprising that this variation was not seen for the women, but we have no comparison for women with respect to geographical location. Other studies have also found a difference in tHcy levels between a high-CHD-risk area (Belfast) and low-risk-areas (in France) [19], and again the exact reason for this was not established.

There was a profound negative correlation between tHcy levels and folate in the present study, which has been found in most other studies [2]. There is documented evidence that dietary folate intake through fruits and vegetables varies geographically in Britain, with the intake being lower in Scotland than in the South of England (Ministry of agriculture, 1996) [20]. This is, however, not reflected in differences in the levels of serum folate or vitamin B_{12} between men in the two towns in the present study, and differences in tHcy levels between the two towns were not affected by adjustment for folate and vitamin B_{12}. Although smoking has a profound effect on levels of tHcy [21], adjust

Table 5
Total serum homocysteine (age and sex adjusted geometric mean, 95% CI) in subjects with combined C677T and A1298C genotypes

677(C-T)/1298(A-C)	CC	AC	AA
CC	11.3 (10.3, 12.3) $N = 43$	11.2 (10.7, 11.7) $N = 159$	11.0 (10.5, 11.6) $N = 134$
CT	12.0 (10.0, 14.4) $N = 10$	11.6 (11.0, 12.1) $N = 137$	11.2 (10.7, 11.7) $N = 185$
TT	9.8 (6.4, 14.8) $N = 2$	11.4 (9.7, 13.4) $N = 13$	12.9 (12.0, 14.0) $N = 56$

ment for smoking differences between towns did not alter the significance of the difference in tHcy between the towns. The effect of smoking on levels of tHcy is consistent with other studies, such as the US Physicians' Health Study [21], and the Hordaland Homocysteine Study [22]. Furthermore, in the US Physicians' Health Study [21], smoking was a significant determinant of plasma levels of folate, as was the case in the present study. Despite this, the levels of folate do not differ between the two towns as a whole. This is also consistent with the analysis carried out on the whole BRHS cohort, where smoking did not explain the geographic variation or the gradient in tHcy levels [3].

The MTHFR genotype provides a possible explanation of the observed town difference in levels of tHcy. All the subjects were genotyped for the two polymorphisms in the MTHFR gene. The frequency of the 677T allele, 0.32 overall, is similar to the frequency of 0.34 previously observed in the UK population [11]. However, when the frequency of the T allele was examined in the two towns, there was a highly statistically significant difference between Maidstone (0.29) and Dewsbury (0.35), $P < 0.01$. The frequency of the 677T allele is known to vary between countries [11] and even within a country [23], but the frequency difference observed between towns in this study raises the possibility of a sampling bias. When the frequency was examined separately in men and women, the same town difference was seen for both genders, lending support to a true regional or town difference in the frequency of the 677T allele. As a frequency difference has a great potential for bias, when comparing allele frequencies in genetic studies of disease, further studies on frequencies of the 677T allele in the MTHFR gene within the UK and other countries are warranted. The frequency of the less common allele for the A1298C polymorphism was not statistically significant between towns or between men and women. The frequency reported here (0.28) is slightly lower than that reported previously [12] i.e. (0.33), and may be explained by the fact that allelic frequencies are population specific.

When the effect of the C677T polymorphism on levels of tHcy was examined, the consistently-observed effect of the 677TT homozygotes to have higher levels of tHcy [10] was seen in the sample as a whole ($P < 0.001$). However, when men and women were examined separately, the effect was much more profound in the men. Most studies, examining the effect of this polymorphism on levels of tHcy have been in men, but previous studies of women have shown the same effect [24]. When the distribution of the 677TT homozygotes was examined, the highest proportion was seen in the highest quartile of tHcy, but only for the men (1.96 vs. 14% in the lowest and the highest tHcy quartile respectively, $P < 0.02$). In the women, on the other hand, the distribution of the 677TT homozygotes was more even over all the quartiles, suggesting that a gender associated factor must be modulating or abolishing the effect of 677TT homozygosity on blood levels of tHcy. It is well documented that folate reduces or abolishes the tHcy raising effect of the 677TT alleles. This may well be a major contributing factor in this study as homocysteine is strongly inversely related to folate levels in all quartiles of tHcy concentration. The use of hormone replacement therapy (HRT) is also known to affect blood levels of tHcy [25,26] but with inconsistently lasting effect [25] and the least effect in women of the 677TT genotype [27]. The average age of the women in this study was nearly 66 years and most of them are, thus, likely to be postmenopausal and may well be on HRT, although no information was available on menopausal status or HRT of the women in this study. Fasting and post-methionine load tHcy concentrations have been reported to be much lower in premenopausal women than in men of the same age as well as in postmenopausal women. Boers et al. [28] suggested that premenopausal women have greater efficiency in handling methionine loading and they speculated that this phenomenon might account for the lower incidence of vascular disease in women during their reproductive lives. By contrast, Anderson et al. [29] measured both free and total plasma homocysteine levels before and after methionine loading in men, premenopausal and postmenopausal women and found no gender or menopausal status differences in fasting or post-methionine loading homocysteine levels.

It is unlikely that this lack of effect of the C677T polymorphism on tHcy levels in women explains the large difference in tHcy levels between men and women in this study as the difference was still statistically significant after adjustment for the MTHFR genotype. This was also the case after adjustment for vitamin B_{12} levels that were significantly different between the genders in this sample. Thus, there is no clear explanation for this difference between men and women in levels of tHcy.

Despite considerable linkage disequilibrium with the C677T polymorphism, no effect on levels of tHcy or folate and vitamin B_{12} was seen when the effect of the A1298C polymorphism was examined in this sample. Van der Put et al. [12] reported that neither the homozygous nor the heterozygous state of the A1298C genotype was associated with higher tHcy or lower folate levels in a sample of neural tube defect patients and their parents. Our results confirm these findings in a representative general population. However, Van der Put [12] suggested that combined heterozygosity for both polymorphisms resulted in reduced MTHFR specific activity, higher tHcy levels and decreased plasma folate, and therefore, combined heterozygotes behaved in a similar manner as those homozygotes for the thermolabile variant. In this sample, when genotypes of

both polymorphisms were combined and tHcy levels were examined, there was no significant interaction between the two mutations and combined heterozygotes for both mutations did not have significantly higher tHcy levels when compared with any other genotype.

The differences in tHcy levels between men in the two towns might possibly be explained by the MTHFR genotype. However, the difference in tHcy was still observed after adjusting for the C677T polymorphism. The frequency difference of the 677T allele between the two towns is likely to be a true difference as discussed above. Few studies have described differences in tHcy levels between towns or regions, or studied the effect of genetic variation on the difference. We have reported [11] a difference in levels of tHcy between countries, where a significantly higher level of tHcy was seen in Italy compared with other European countries. This coincided with a much higher frequency of the 677T allele, and in particular with higher proportion of 677TT homozygotes, similar to the observation in the study described here. However, as in the present study, the difference in tHcy levels was not explained by the higher frequency of the 677T allele, using the same conventional statistical analysis. This leaves the possibility that there is another, yet unidentified, environmental or genetic factor, responsible for the higher tHcy levels. For the genetic factor, this could be a genetic variation in linkage disequilibrium with the thermolabile C677T polymorphism in the MTHFR gene, or in an altogether different gene, involved in the metabolism of homocysteine. It is potentially of great importance to examine further this possible association between regional differences in blood levels of tHcy, coinciding with frequency differences of the C677T polymorphism in the MTHFR gene.

In this sample of older adults, the effect of the C677T polymorphism on blood levels of tHcy is statistically significant, although it only explains just under 2% of the total variance in levels of tHcy. In a previous study of young individuals [11], this same polymorphism explained about 12% of the total variance in plasma tHcy concentration. The smaller value observed in these elderly subjects, suggests that environmental factors, probably mainly diet or possibly increasing age or age-related life-style factors, are the largest determinant of tHcy levels. However, although in these groups of healthy subjects environmental factors have the major impact on the blood levels of tHcy, there is still room for one or more genetic components to be identified in the determination of tHcy levels.

Acknowledgements

This work was supported by the British Heart Foundation, RG-950007, PG95/189 and FS/97049. The British Regional Heart Study is a British Heart Foundation Research Group and also receives support from the Department of Health and the Stroke Association.

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