# Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia

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**Low blood folate levels result in hyperhomocysteinemia, which has been associated with increased risk for cardiovascular disease, neural tube defects and cognitive deficits. Intake of dietary folates is the chief determinant of blood folate levels. Molecular defects in the intestinal absorption of dietary folates that precipitate low blood folate levels and hyperhomocysteinemia have not been investigated previously. Dietary folates are a mixture of polyglutamylated folates which are digested to monoglutamyl folates by the action of folylpoly-γ-glutamate carboxypeptidase (FGCP), an enzyme that is anchored to the intestinal brush border membrane and is expressed by the glutamate carboxypepidase II (*GCPII*) gene. We cloned *GCPII* cDNA from human intestine and identified both a full-length transcript and a 93 bp shorter transcript lacking exon 18, consistent with the presence of a splice variant. In addition, we identified an H475Y polymorphism in *GCPII* in DNA samples from a healthy Caucasian population (*n* = 75). We found that membranes of transfected COS-7 cells expressing the H475Y variant *GCPII* cDNA had 53% less FGCP activity than did cells expressing wild-type *GCPII*. The presence of the H475Y *GCPII* allele was significantly associated with lower folate and higher homocysteine levels in this population. These data suggest that the presence of the H475Y *GCPII* allele impairs the intestinal absorption of dietary folates, resulting in relatively low blood folate levels and consequent hyperhomocysteinemia.**

## INTRODUCTION

Folates are a family of vitamins that are required for DNA synthesis and the transfer of methyl groups in the methionine cycle (1). Low folate status induces alterations in methionine metabolism that result in hyperhomocysteinemia (2,3) which has been associated with increased risk of cardiovascular disease (4), cognitive deficit in the elderly (5–9) and neural tube defects (10). Blood folate levels are regulated in part by the intestinal absorption of dietary folylpoly-γ-glutamates, the predominant form of naturally occurring dietary folates. The absorption of dietary folates involves the initial hydrolysis of terminal glutamate residues of folylpoly-γ-glutamates by folylpoly-γ-glutamate carboxypeptidase (FGCP), an exopeptidase which is anchored to the intestinal apical brush border membrane. The hydrolytic step is followed by membrane transport of monoglutamyl folate derivatives by a separate membrane protein, the reduced folate carrier (11). FGCP may regulate the availability of dietary folates, since folylpoly-γ-glutamates are taken up by the perfused human jejunum at two-thirds of the efficiency of similar concentrations of monoglutamyl folic acid (11,12).

Purified FGCP from pig and human jejunal brush border membranes is a zinc-activated exopeptidase that hydrolyzes the terminal glutamate residues of folylpoly-γ-glutamates (12–14). The nucleotide and predicted amino acid sequences of pig jejunal FGCP cDNA (15) are 88 and 92% identical to those of human prostate-specific membrane antigen (PSM), a marker for prostate cancer (16) and 83% identical to rat *N*-acetylated α-linked acidic dipeptidase (NAALADase) (17), a brain enzyme which regulates glutamate neurotransmission by hydrolyzing the glutamate residue of *N*-acetyl-aspartyl-glutamate. The recent molecular characterization of human brain NAALADase cDNA confirmed its sequence identity to PSM (18). Purified pig jejunal brush border membranes and membranes of human prostate cancer PC3 cells transfected with the cDNA of pig FGCP exhibited both FGCP and NAALADase activity (15), consistent with previous findings that PSM is capable of hydrolysis of glutamate residues of folylpoly-γ-glutamates and *N*-acetyl-aspartyl-glutamate (19,20). This cumulative evidence supports the concept that FGCP, NAALADase and PSM are functionally distinct expressions of a single gene that encodes 750 amino acids and is collectively known as glutamate carboxypeptidase II (GCPII). The genomic sequence of *GCPII* (characterized as PSM) is comprised of 19 exons (GenBank accession no.

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AF007544) and has been localized to chromosome 11p11.2 (21,22). Structural analysis of the predicted amino acid sequence of GCPII showed similarities with the M28 peptidase family and identified a single N-terminal membrane-spanning region and a catalytic region of 313 amino acids, which contains two co-catalytic zinc atoms (23). This model was substantiated by the demonstration that NAALADase activity is reduced by site-directed mutagenesis of the predicted zinc-binding sites of GCPII (24).

We propose that low levels of folate and higher levels of homocysteine in the blood may result from a molecular defect in GCPII that results in decreased absorption of dietary folates. Low levels of circulating folate are associated with decreased folate-dependent conversion of homocysteine to methionine and would predictably result in elevated levels of serum homocysteine. To address this hypothesis, our first objective was to isolate GCPII from human intestine and characterize its molecular structure including identification of potential functional polymorphisms that affect the activity of FGCP in cell culture. Our second objective was to correlate the incidence of a functional polymorphism of GCPII with relatively lower folate levels and hyperhomocysteinemia in a healthy Caucasian population (7).

## RESULTS

### Characterization of human intestinal GCPII

To characterize GCPII from human intestine and to confirm its identity with cDNAs of human PSM and NAALADase, we screened two commercial human intestinal cDNA libraries with an 852 bp PCR fragment of the open reading frame (ORF) of pig jejunal FGCP cDNA and identified two overlapping clones. Sequence analysis showed that these two clones were 100, 88 and 83% homologous to corresponding regions in human PSM cDNA (16), pig FGCP cDNA (15) and rat NAALADase cDNA (17), respectively, with the exception of a 93 bp deletion in the 3′ end. Further sequence analysis using BLASTN (25) identified the 93 bp deletion as exon 18. Both the splice variant and the wild-type transcripts of GCPII were identified by RT–PCR of normal human jejunal mucosa obtained at elective gastric bypass surgery in an obese patient. Subsequently, we confirmed the presence of both wild-type and splice variant forms of GCPII by RT–PCR consistently in 40 intestinal biopsy samples from human subjects undergoing diagnostic endoscopic procedures (26). Analysis using rapid amplification of cDNA ends (RACE) recovered an additional 83 bp of the 5′ untranslated region (UTR) of intestinal GCPII that placed the transcriptional start site at –138 relative to the translation initiation codon.

A commercial multiple human tissue northern blot (Clontech) was probed with human intestinal GCPII cDNA to determine the tissue distribution of GCPII mRNA. A predominant 2.8 kb band was identified in prostate, small intestine, brain, kidney, liver, spleen and colon, listed in order of decreasing intensity (Fig. 1). A less prominent 1.5 kb band was also identified in liver and kidney.

### Polymorphism analysis of GCPII

To identify a potentially significant polymorphism in GCPII associated with low folate levels and hyperhomocysteinemia,
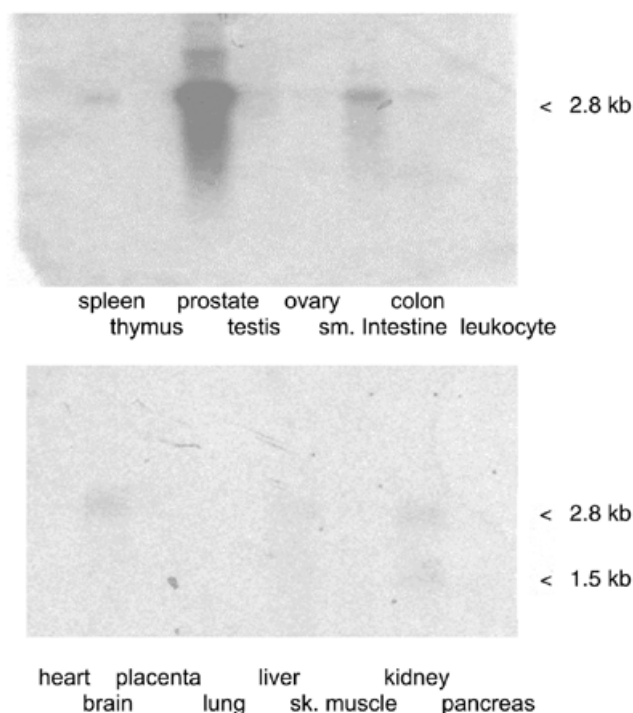


**Figure 1.** Northern blot showing tissue distribution of GCPII transcripts. Human multiple tissue northern blots were incubated with pFGCP52 labeled with [α-$^{32}$P]dCTP. A 2.8 kb band predominates in the prostate, small intestine, brain, liver and kidney with a less intense band in spleen and colon. A second 1.5 kb band is also present in the liver and kidney.

we screened for GCPII polymorphisms in a subset of 75 stored DNA samples obtained retrospectively from healthy Caucasian subjects (age >50 years) residing in Oxford, UK, who participated as a control group in a study of folate and homocysteine levels in Alzheimer's disease (7). PCR primers were designed on the basis of the genomic sequence of human GCPII (21) in order to amplify the exon–intron borders and 19 exons of human GCPII (Fig. 2). Initially we amplified all exons in 20 DNA samples followed by polymorphism screening using temperature modulated heteroduplex chromatography (27) and identified a polymorphism in exon 13. Subsequent sequence analysis of the PCR product of exon 13 identified a polymorphism, characterized by a C→T single base substitution at nucleotide 1561 corresponding to codon 475, predicted to replace a histidine with tyrosine (Fig. 2). This polymorphism is located in the putative catalytic region of GCPII (23). The structural organization of human GCPII based on its genomic sequence and prior analysis (21,23) is shown in Figure 2.

We next determined *in vitro* the functional significance of the H475Y variant in relation to wild-type GCPII. Mammalian COS-7 cells, predetermined to lack endogenous FGCP activity, were transfected with plasmids containing wild-type GCPII cDNA or H475Y GCPII cDNA, prepared as described in Materials and Methods. Compared with FGCP activity in wild-type GCPII COS-7 transfectants, the H475Y GCPII variant resulted in a significant 53% reduction ($P < 0.01$) in FGCP activity (Fig. 3a). Western blot analysis using the monoclonal antibody J591 against the extracellular domain of GCPII (28) (a gift from Dr N. Bander, Cornell University) identified a
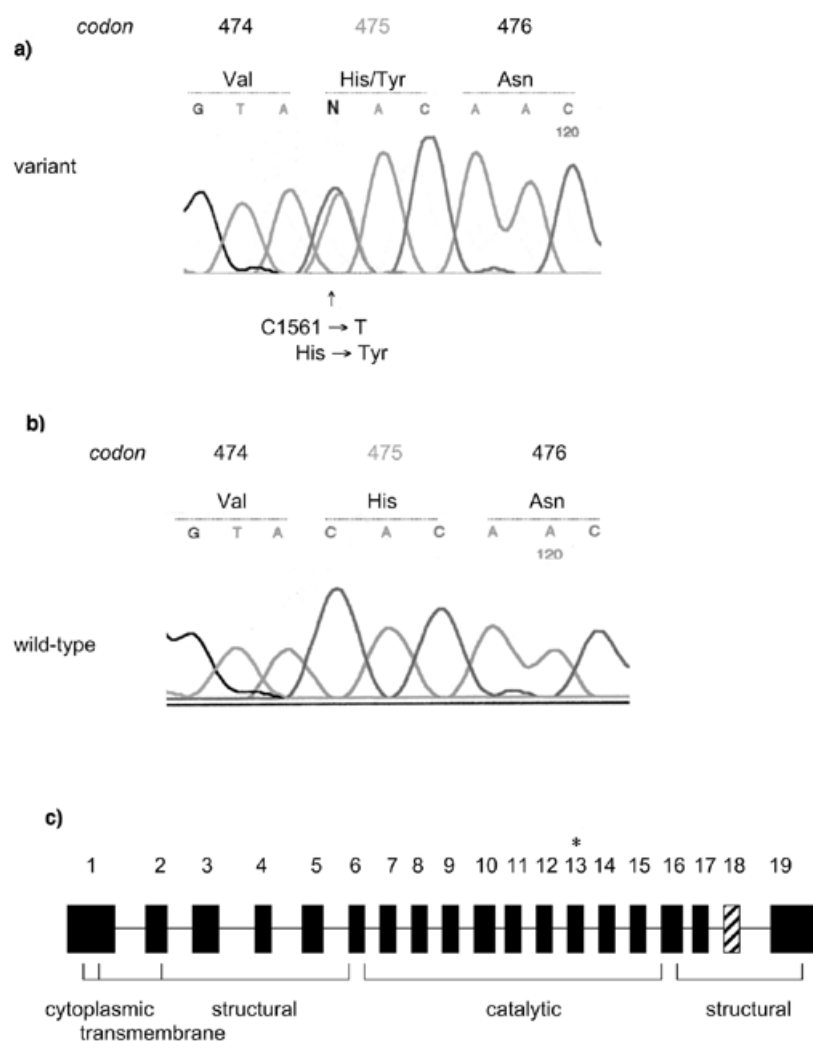
**Figure 2.** The DNA sequence of a portion of exon 13 obtained from amplification by PCR of genomic DNA revealing the exon 13 polymorphism. (**a**) An electropherogram showing the sequence from a portion of exon 13 that revealed the C→T substitution at nucleotide 1561 (GenBank accession no. AF176574) corresponding to codon 475 predicted to replace a histidine with tyrosine from a subject heterozygous for the polymorphism. (**b**) An electropherogram showing the wild-type sequence from a portion of exon 13. (**c**) Proposed exon map of human *GCPII* based on the genomic sequence of *GCPII* (21). The H475Y *GCPII* polymorphism is localized to exon 13 as shown by an asterisk. The exon 18 deletion resulting from the splice variant is shown with hatched bars. The predicted regions coding for the various functional domains are based on the Rawlings and Barrett analysis (23).

110 kDa band in isolated human jejunal brush border membranes and in membranes from COS-7 transfectants expressing either wild-type *GCPII* or the H475Y variant of *GCPII* (Fig. 3b).

We designed a PCR restriction enzyme method to genotype all the DNA samples. The C→T substitution in codon 475 creates an *Acc*I site (Fig. 4). Amplification of exon 13 by PCR yields a 244 bp fragment, which is cut by *Acc*I to produce 141 and 103 bp bands if the polymorphism is present (Fig. 4). We used PCR followed by digestion with *Acc*I to screen the 75 stored DNA samples for the H475Y *GCPII* polymorphism, followed by sequence confirmation of positive samples. This process yielded six heterozygous variants and no homozygous variants. Of interest, all six subjects who had the H475Y *GCPII* allele were women. However, we cannot assume gender specificity since the sample was skewed towards women (44 of 75) and combined analysis of this group with a group of Alzheimer's disease patients (7) showed equal

distribution of the allele among both genders (29). The overall allele frequency of the H475Y *GCPII* polymorphism in the 75 healthy subjects was 0.04, with a genotype frequency of 8% heterozygote and 92% homozygote wild-type. According to the Hardy–Weinberg Law, the predicted frequency of homozygous genotype for the H475Y *GCPII* polymorphism in this population is 0.0016 or 1 in 625 subjects.

### Relationship of the H475Y *GCPII* polymorphism to folate status and hyperhomocysteinemia

Among the 75 healthy individuals with available DNA from the previous study (7), H475Y-positive subjects had significantly lower serum folate levels ($P < 0.05$) and higher serum homocysteine levels ($P < 0.05$) than wild-type subjects (Table 1). There was no relationship between the presence of the predetermined 677C→T methylenetetrahydrofolate reductase (MTHFR) allele (7) and H475Y *GCPII*. There was no signifi-
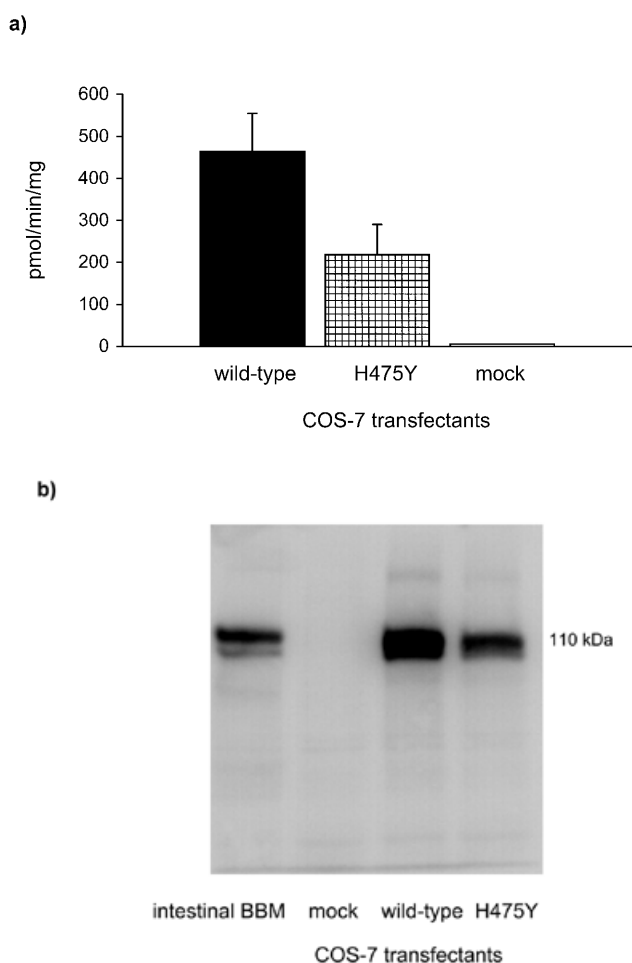
a)



b)



**Figure 3.** GCPII protein expression and FGCP activity in membranes of COS-7 cell transfectants. (**a**) FGCP activity was determined in membranes from COS-7 cell transfectants using the substrate folyl-γ-glutamyl-γ-[$^{14}$C]glutamate (13,31). COS-7 cells were transfected with constructs of either *GCPII* cDNA (wild-type) or H475Y variant *GCPII* cDNA (H475Y) in pTRACER-CMV2. Cells were also transfected with pTRACER-CMV2 (mock) as a control. Results show the means + SD of three experiments for each transfectant. There was no activity in mock-transfected cells. FGCP activity was significantly 53% lower ($P < 0.01$) in membranes from H475Y *GCPII* variant transfectants than membranes from wild-type *GCPII* transfectants. (**b**) Western blot analysis. Using the monoclonal antibody J591 against the extracellular domain of GCPII (44; a gift from Dr N. Bander), GCPII was detected in membranes of COS-7 cell transfectants expressing wild-type and the H475Y variant of GCPII.

cant relationship of the H475Y *GCPII* allele with red blood cell (RBC) folate or vitamin B$_{12}$ levels, whereas RBC folate levels were significantly associated ($P < 0.05$) with vitamin B$_{12}$ levels. Homocysteine varies by gender, age and by differences in blood levels of vitamin B$_{12}$, folate and creatinine (28–30). After controlling for gender, age and blood levels of vitamin B$_{12}$ and creatinine, the relationship of the H475Y *GCPII* allele remained positively associated with elevated homocysteine levels ($P < 0.02$). Serum folate was the only variable that influenced the relationship between *GCPII* genotype and homocysteine levels ($P < 0.0001$). These findings suggest that the significant association of higher levels of homocysteine

**Table 1.** Distribution of folate, vitamin B$_{12}$, homocysteine and creatinine according to *GCPII* genotype (means ± SEM)

| Clinical parameter | *GCPII* genotype | |
|---|---|---|
| | HY | HH |
| Serum folate (μg/l) | 7.0 ± 1.2[a] ($n = 6$) | 10.9 ± 0.5 ($n = 69$) |
| RBC folate (μg/l) | 463 ± 95.5 ($n = 6$) | 471 ± 21.1 ($n = 67$) |
| Vitamin B$_{12}$ (ng/l) | 347 ± 51.0 ($n = 6$) | 470 ± 28.9 ($n = 69$) |
| Homocysteine (μmol/l) | 15.0 ± 1.10[b] ($n = 6$) | 12.6 ± 0.93 ($n = 54$) |
| Creatinine (μmol/l) | 86.8 ± 7.22 ($n = 6$) | 94.1 ± 2.40 ($n = 68$) |

HY, heterozygous for H475Y *GCPII* polymorphism; HH, wild-type GCPII.
[a]$P < 0.05$, [b]$P < 0.02$, comparing values according to genotype.

with the H475Y allele is a consequence of the effect of serum folate on homocysteine levels.

## DISCUSSION

We characterized the cDNA sequence of human intestinal *GCPII* and found near complete identity to human PSM (16) and NAALADase (18). This study confirms prior evidence that human *GCPII* is expressed as three functionally distinct proteins, namely: intestinal FGCP, brain NAALADase (17,18) and PSM (16). The transcriptional start site for *GCPII* in human jejunum was determined by RACE at nucleotide –138 relative to the translation initiation codon. This finding contrasts the multiple transcriptional start sites determined for PSM in prostate cancer cells at nucleotides –262, –235 and –195 relative to the translation initiation codon (16,21). The differences in start sites between intestine and prostate suggest that transcriptional regulation of GCPII is governed in a tissue-specific fashion. The present results show also that the 5′ UTR of intestinal *GCPII* mRNA is shorter than that determined for PSM mRNA (16,21). A PSM splice variant, denoted as PSM′, was identified in human prostate cancer LNCaP cells and characterized as lacking 266 bp of the 5′ region of the PSM cDNA, corresponding to 146 bp of the 5′ UTR and 120 bp of the ORF (30,31). However, it is unlikely that PSM′ is present in the intestine since the 5′ UTR region present in PSM′ is not found in intestinal *GCPII* mRNA. On the other hand, an intracellular enzyme capable of hydrolyzing both internal and terminal γ-glutamate residues of folylpoly-γ-glutamates was previously purified from human intestinal mucosa (32) and is encoded by a separate gene (33). A previous study from our laboratory demonstrated that intestinal brush border FGCP and not the intracellular enzyme functions in the digestion of folylpoly-γ-glutamates in the lumen of pig intestine (14).

The identification of a *GCPII* splice variant lacking exon 18 in human jejunum is consistent with previous reports describing a similar 93 bp shorter NAALADase transcript in rat (34) and human brain (35). Regulation of the translation and the potential effect of varied amounts of the splice variant on FGCP and NAALADase activities remain to be determined.

Further studies of human *GCPII* expression using northern analysis showed its expression as a predominant 2.8 kb band in prostate, small intestine, brain, liver, kidney and colon (Fig. 1), consistent with previously reported findings of PSM expression in
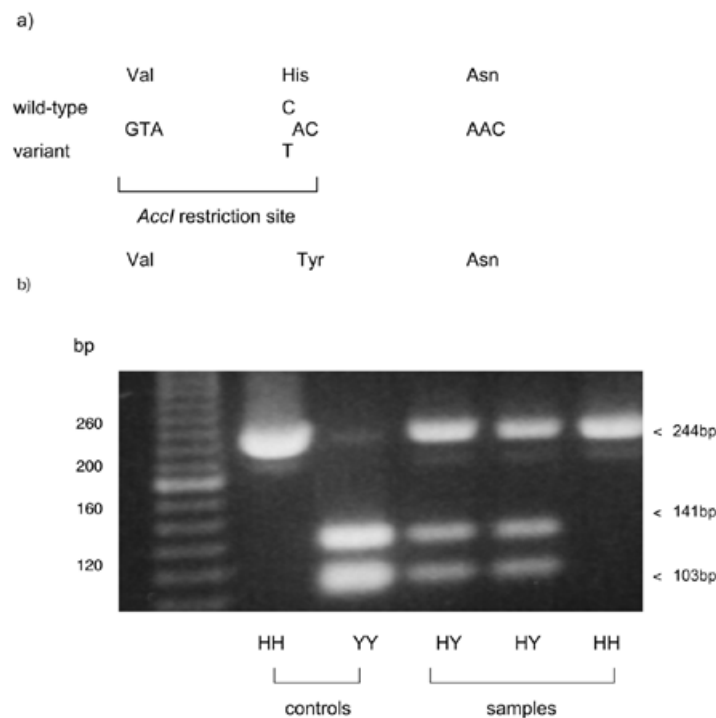
**Figure 4.** (**a**) Amplification of exon 13 by PCR produces a 244 bp fragment. The C→T polymorphism at codon 475 inserts a unique *Acc*I site. (**b**) Agarose gel showing products obtained from amplification of exon 13 followed by digestion with *Acc*I. Digestion of PCR products containing the polymorphism yields 141 and 103 bp fragments whereas *Acc*I does not cut wild-type PCR products.

prostate cancer cells (16) and NAALADase expression in brain (17,18). We also identified a less predominant *GCPII* band at 1.5 kb in the kidney and liver, which has not been reported previously for PSM or NAALADase. The significance of this smaller transcript remains to be determined but may represent tissue-specific splice variants or products of *GCPII* mRNA degradation. These findings confirm the multiple tissue expression of *GCPII* and its expression in prostate, small intestine and brain (15–18). Further studies are required to determine the horizontal (proximal to distal) and vertical (crypt to villus) distribution of *GCPII* expression in human small intestinal epithelial cells.

Using DNA samples from a previous study of Caucasian subjects (7), we identified a C→T polymorphism predicted to replace a histidine with a tyrosine at codon 475 in exon 13 of the catalytic region of GCPII (Fig. 2). The functional significance of the polymorphism was shown by findings *in vitro* that the H475Y *GCPII* cDNA expressed 47% of the activity of wild-type FGCP in membranes of transfected COS-7 cells (Fig. 3). Western blot analysis revealed that cell membranes expressing the H475Y variant exhibited GCPII protein with a band intensity that was somewhat less than that observed in membranes of cells expressing wild-type GCPII. Since the J591 antibody binds to the extracellular region of GCPII (28) that also contains the H475Y polymorphism, the lower band intensity observed for cell membranes expressing the H475Y variant of GCPII may simply reflect decreased efficiency of the binding of the antibody. Alternatively, the H475Y polymorphism may affect FGCP activity through altered post-translational processing and/or activity due to altered configuration of the catalytic region of the enzyme.

Intestinal FGCP cleaves glutamate residues from folylpoly-γ-glutamates and thus plays an important regulatory role in the intestinal absorption of dietary folylpoly-γ-glutamates (11). Therefore, polymorphisms in GCPII affecting the activity of FGCP would predictably decrease the intestinal absorption of dietary folylpoly-γ-glutamates and consequently decrease folate levels and increase homocysteine levels in the body. To test this hypothesis, we analyzed DNA samples from a subset of healthy control subjects from a previous study of English subjects (7). Our hypothesis was corroborated by the present findings of significantly lower serum folate levels and higher homocysteine levels in the subjects with the H475Y *GCPII* allele than in subjects with wild-type *GCPII* alleles (Table 1). These findings are consistent with the concept that the H475Y *GCPII* allele affects folate absorption, resulting in lower serum folate levels in a healthy population.

Serum folate levels may be influenced by variations in dietary intakes of folates, which were not assessed in the original study of the present cohort (7). During the time when the majority of samples were collected the average daily folate intake for the population in the UK was 233 ± 90 μg/day according to a national survey carried out in 1994/1995 (36) which is less then the current recommended dietary intake of folate at 400 μg/day in the USA (37). Further work is required to determine whether the effect of the H475Y GCPII polymorphism on blood folate and homocysteine levels is influenced by the amount of dietary and/or supplemental folates in a similar way to the effect of the C677T MTHFR polymorphism (38).

That we observed no differences in RBC folate levels according to *GCPII* genotype may be explained by the

confounding effect of vitamin $B_{12}$ levels, which were unrelated to *GCPII* genotype but which affect levels of tissue folates. Methylated monoglutamyl folates must be demethylated and then polyglutamylated by folylpoly-γ-glutamate synthase for intracellular storage in tissues as polyglutamyl folates (1). The demethylation reaction is catalyzed by vitamin $B_{12}$ and, therefore, the ability of RBCs to store polyglutamylated folates is dependent on vitamin $B_{12}$.

Serum homocysteine levels are influenced by nutritional deficiencies of folate, vitamin $B_{12}$ or vitamin $B_6$, by genetic defects in one or more of the proteins involved in the metabolism of methionine and by renal function, age and gender (39–42). The thermolabile 677C→T polymorphism in MTHFR, an enzyme required for the synthesis of methyl tetrahydrofolate, the substrate for the conversion of homocysteine to methionine, has been implicated in the pathogenesis of hyperhomocysteinemia (42). In the present study, the association of the H475Y allele of *GCPII* with higher homocysteine levels was independent of gender, age, renal status and *MTHFR* genotype. Within the entire group, serum folate also influenced the relationship between *GCPII* genotype and homocysteine. This finding implies that the relationship of the H475Y *GCPII* allele with homocysteine levels is mediated by the effect of the variant allele on serum folate levels. Further studies with larger population groups are needed to establish the significance of the H475Y polymorphism in the expression of GCPII in clinically relevant conditions.

## MATERIALS AND METHODS

### Cloning of human intestinal FGCP cDNA

Two separate human jejunal cDNA libraries in pcDNA2 (Invitrogen, Carlsbad, CA; a gift of B. Lonnerdal, University of California, Davis, CA) and λgt10 (Clontech, Palo Alto, CA; a gift of H. Said, University of California, Irvine, CA) were screened with an [α-$^{32}$P]dCTP-labeled 852 bp PCR fragment of the ORF of the pig jejunal FGCP cDNA (15). Standard protocols were followed for library screening (43). Two positive clones were identified and sequenced using the LI-COR 4200 automated sequencer (LI-COR, Lincoln, NE). The transcriptional start site was determined by 5′ RACE using the Marathon Ready human small intestine cDNA and the Marathon RACE kit (Clontech). The first PCR reaction used the gene-specific primer 5′-GTGCTAGCTCAACAGAATCCAGGC-3′ followed by a second PCR reaction with the nested gene-specific primer 5′-CCACAGCCGAGTCGGTTTCGTGAA-3′. Comparative analysis with the GenBank nucleic acid database using BLASTN (25) revealed that clones pFGCP52 and pFGCP72 were completely homologous to the human PSM cDNA sequence (16) except for a 93 bp deletion in the 3′ end of clone pFGCP52. Further sequence analysis with PSM genomic sequence (21) revealed that the 93 bp deletion represented exon 18. The presence of the splice variant in human intestine was confirmed by RT–PCR of human jejunal mucosal RNA. A specimen of normal adult human jejunum was obtained at surgery from an obese patient undergoing elective gastric bypass with construction of gastro-jejunal anastomosis. The jejunal mucosa was scraped with a glass slide then immediately placed in TRIzol reagent (Life Technologies, Grand Island, NY), frozen in liquid nitrogen and stored at –80°C until RNA

extraction. Total RNA was extracted using TRIzol reagent followed by reverse transcription using Superscript reverse transcriptase (Life Technologies) and PCR of the first strand cDNA using primers flanking exon 18, forward in exon 16 (5′-GAAACAAACAAATTCAGCGG-3′) and reverse in exon 19 (5′-AGAGCATCATAAATTCCTGG-3′). BLASTP (25) was used to compare the predicted amino acid sequence of human intestinal *GCPII* cDNA with the GenBank protein database.

### Northern analysis

Selected human multiple tissue northern blots of mRNA were obtained from a commercial source, each standardized to actin (Clontech) were hybridized at 42°C for 18 h with clone pFGCP52 labeled with [α-$^{32}$P]dCTP using the RadPrime DNA labeling system (Life Technologies). Following hybridization and washing, the blots were exposed to X-OMAT AR film (Eastman Kodak, Rochester, NY) for 48 h with intensifying screens at –80°C.

### Human *GCPII* polymorphism analysis

Genomic DNA samples were obtained retrospectively from 75 Caucasian subjects, aged 50 years and older, who participated as control subjects in a previously published study of folate metabolism in Alzheimer's disease (7). Exons and exon–intron borders of *GCPII* were amplified in each DNA sample using the following primers designed from the reported genomic PSM sequence (21):
exon 2, 5′-GTATCTCATGCCTATTTTTGG-3′
and 5′-GTCCATATAAACTTTCGAGGA-3′;
exon 3, 5′-CCACTTTCAATTTTTGTTCACC-3′,
5′-ACATCATAATGAGCTAGCTCAA-3′,
5′-CCTGTTGTCCTACCCAAA-3′
and 5′-AAATGGGGGGAATGTTTC-3′;
exon 4, 5′-GTAATCAAACTTTAACTG-3′
and 5′-GTCATTTTTATTTGTTGC-3′;
exon 5, 5′-AAGTCATTCCCGATCTCAA-3′
and 5′-AACTTTTTACATTGGGGGA-3′;
exon 6, 5′-AAAGACACTGTGTTTTCTAG-3′
and 5′-AAGTTTCCTATGATATTCAA-3′;
exon 7, 5′-AACTGAGTTTTTACTGGAAA-3′
and 5′-ACTGTCAATAAGAAATTAC-3′;
exon 8, 5′-AACATTTTAATGCGTTACTTCAA-3′
and 5′-CCCCTTAAAAGAGTTAAAATTAA-3′;
exon 9, 5′-AATTCCCTTAAACCTTATAG-3′
and 5′-ACTTGATCAATATTTGCTAA-3′;
exon 10, 5′-AATCTTGTATCTATGCTTTCTAG-3′
and 5′-AGAAGACTAAACTGAGACTCAG-3′;
exon 11, 5′-GGGCTTGGTAGTGTCCTGG-3′
and 5′-GCTTGGCAAACAAGTCCTGG-3′;
exon 12, 5′-AATCTACATACATTAAATGTAG-3′
and 5′-AGTGCATAATTTATTAACTAGA-3′;
exon 13, 5′-CATTCTGGTAGGAATTTAGCA-3′
and 5′-AAACACCACCTATGTTTAACA-3′;
exon 14, 5′-AATGGACATCTAGTTTTTAA-3′
and 5′-ACTTAATGATTGAAAGAAAA-3′;
exon 15, 5′-AAGAATGGGGTTTAGTTTAA-3′
and 5′-AGTCAGAATTATTTGTTTAG-3′;
exon 16, 5′-AATAGTAAATATGG TGCTAA-3′,
5′-ACAGTGAGGTGATATTTAAA-3′,
5′-AAATATCACCTCACTGTGG-3′

and 5′-AATAGAACCATACAGATGAG-3′;
exon 17, 5′-AAGCATTTGATGATC CCAA-3′
and 5′-AAAGCTAGTTCAGCAACAG-3′;
exon 18, 5′-GGTAGAAACATATTTTCATGAA-3′
and 5′-AAAAAGTGATATTACAGAAAGG-3′;
exon 19, 5′-GGGC ACCTAATAAACAGCAAA-3′
and 5′-GGCTACTTCACTCAAAGTCTC-3′.

Each PCR product was screened for polymorphisms by temperature-modulated heteroduplex chromatography (27) using the WAVE DNA Fragment Analysis System (Transgenomic, San Jose, CA). PCR samples found to contain polymorphisms and representative wild-type samples were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and both strands were sequenced using the PCR primers and an ABI Prism 377 DNA automated sequencer.

## Biochemical analyses

Non-fasting blood samples were obtained in the period 1988–1995 as described previously (7). Serum and RBC folate levels were determined by microbiological assays with *Lactobacillus casei* in the Clinical Chemistry Labaoratory of the Radcliffe Infirmary, Oxford University, Oxford, UK. Homocysteine levels were determined by high-performance liquid chromatography with fluorescence detection (44). Vitamin $B_{12}$ levels were measured by radioimmunoassay.

## Transfection studies

COS-7 cells (ATCC, Rockville, MD) were transfected with DNA constructs of the wild-type *GCPII* cDNA or *GCPII* cDNA containing the H475Y polymorphism. The cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin (Life Technologies). The cells were grown in at 37°C in a 5% $CO_2$ incubator. The wild-type *GCPII* cDNA was synthesized by PCR of reverse transcribed human jejunal mucosal RNA using *Pfu* polymerase (Stratagene, La Jolla, CA) and primers at the 5′ end based on the RACE sequence and the 3′ end based on the clone pFGCP52 sequence. The wild-type *GCPII* cDNA was cloned into the mammalian expression vector, pTRACER-CMV2 (Invitrogen). The H475Y polymorphism was introduced into *GCPII* cDNA in pTRACER-CMV2 using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The primers used in the PCR reaction were 5′-CCGCTGATGTACAGCTTGG-TATACAACCTAACAAAAG-3′ and 5′-CTTTTGTTAGGT-GTATACCAAGCTGTACATCAGCGG-3′. The resulting product was transformed into XL1-Blue cells. Both the full-length wild-type *GCPII* cDNA and mutated *GCPII* cDNA constructs were sequenced using an ABI Prism 377 DNA automated sequencer (PE Biosystems, Foster City, CA). Transfections of COS-7 cells with wild-type and the H475Y variant forms of *GCPII* were accomplished using LipofectAMINE and OPTI-MEM I medium (Life Technologies) according to the manufacturer's protocol. One day prior to transfection, the cells were seeded onto 6-well plates and grown in serum-containing DMEM so that on the day of transfection the cells were 60–80% confluent. Cell membranes were harvested at 48 h post-transfection by scraping into 50 mM Tris–HCl (pH 7.4) followed by homogenization and removal of supernatant after centrifugation, 35 000 *g* for 30 min. The complete membrane pellets were resuspended in 50 mM Tris–HCl and the protein concentration of the membranes was determined using the enhanced protocol BCA assay (BioRad, Hercules, CA). Expression of green fluorescence protein was used to monitor transfection efficiency and uniformity. Similar transfection efficiencies were observed for cells transfected with either wild-type *GCPII* or H475Y variant *GCPII*. Mock-transfected cells served as controls.

## FGCP enzyme activity

FGCP activity was determined in COS-7 transfectant membranes using 12 μM folyl-γ-glutamyl-γ-[$^{14}$C]glutamate substrate and a modification (14) of the method of Krumdieck and Baugh (45). As described previously, this method uses a saturable concentration of substrate and exhibits identical kinetics in purified human and pig intestinal FGCP (13,14). The method was validated previously for measurement of FGCP activity in membranes of PC-3 cells transfected with pig intestinal GCPII, in which we found similar $K_m$ values for transfectant membranes and purified pig jejunal brush border membranes (15).

## Western blot analysis

Human jejunal brush border membranes were isolated as described previously (14). Membranes from cell transfectants (25 μg protein) were resolved on a 7.5% Tris–HCl SDS–polyacrylamide gel (BioRad) and transferred to PDVF membranes (Millipore, Bedford, MA). Immunodetection was performed using the J591 monoclonal antibody against the extracellular domain of GCPII (28) (gift of Dr N. Bander, Cornell University) followed by a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Bands were detected using the SuperSignal West Dura Extended Duration Substrate (Pierce) and a CCD camera (NucleoTech, Hayward, CA) that detects chemiluminescence.

## Statistical analyses

Two-sample Student's *t*-test was used to determine whether the presence of the H475Y *GCPII* allele had an effect on serum folate, RBC folate, vitamin $B_{12}$ and homocysteine. One-way analysis of covariance was use to further analyze the association of the H475Y *GCPII* allele with homocysteine levels controlling for the potentially confounding variables of gender, age, vitamin $B_{12}$ and creatinine.

# REFERENCES

1. Selhub, J., and Rosenberg, I.H. (1996) Folic acid. In Ziegler, E.E. and Filer, L.J. (eds), *Present Knowledge of Nutrition.* ILSI Press, Washington, WA, pp. 206–219.

2. Clarke, R., Woodhouse, P., Ulvik, A., Frost, C., Sherliker, P., Refsum, H., Ueland, P.M. and Khaw, K.-T. (1998) Variability and determinants of total homocysteine concentrations in plasma in an elderly population. *Clin. Chem.*, **44**, 102–107.

3. Selhub, J., Jacques, P.F., Wilson, P.W.F., Rush, D. and Rosenberg, I.H. (1993) Vitamin status and intake as primary determinants of homocysteinemia in an population. *J. Am. Med. Assoc.*, **270**, 2693–2698.

4. Boushey, C.J., Beresford, S.A.A., Omenn, G.S. and Motulsky, A.G. (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *J. Am. Med. Assoc.*, **274**, 1049–1057.

5. Joosten, E., Lesaffre, E., Riezler, R., Ghekiere, V., Dereymaeker, L., Pelemans, W. and Dejaeger, E. (1997) Is metabolic evidence for vitamin B-12 and folate deficiency more frequent in patients with Alzheimer's disease? *J. Gerontol.*, **52**, M76–M79.

6. Nilsson, K., Gustafson, L., Faldt, R., Andersson, A., Brattstrom, L., Lindgren, A., Israelsson, B. and Hultberg, B. (1996) Hyperhomocysteinemia—a common finding in a psychogeriatric population. *Eur. J. Clin. Invest.*, **126**, 853–859.

7. Clarke, R., Smith, A.D., Jobst, K.A., Refsum, H., Sutton, L. and Ueland, P.M. (1998) Folate, vitamin B₁₂, and serum total homocysteine levels in confirmed Alzheimer's disease. *Arch. Neurol.*, **55**, 1449–1455.

8. McCaddon, S., Davies, G., Hudson, P., Tandy, S. and Cattell, H. (1998) Total serum homocysteine in senile dementia of Alzheimer type. *Int. J. Geriatr. Psychiatry*, **13**, 235–239.

9. Selhub, J., Bagley, L.C., Miller, J. and Rosenberg, I.H. (2000) B vitamins, homocysteine, and neurocognitive function in the elderly. *Am. J. Clin. Nutr.*, **71**, 614A–620S.

10. Mills, J.L., McPartlin, J.M., Kirke, P.N., Lee, Y.L., Conley, M.R., Weir, D.G. and Scott, J.M. (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet*, **345**, 149–151.

11. Halsted, C.H. (1990) Intestinal absorption of dietary folates. In Picciano, M.F., Stokstad, E.L.R. and Gregory, J.F. (eds), *Folic Acid Metabolism in Health and Disease.* Wiley-Liss, New York, NY, pp. 23–45.

12. Halsted, C.H., Reisenauer, A.M., Romero, J.J., Cantor, D.S. and Ruebner, B. (1977) Jejunal perfusion of simple and conjugated folates in celiac sprue. *J. Clin. Invest.*, **59**, 933–940.

13. Chandler, C.J., Wang, T.T.Y. and Halsted, C.H. (1986) Pteroylpolyglutamate hydrolase from human jejunal brush borders. *J. Biol. Chem.*, **261**, 928–933.

14. Chandler, C.J., Harrison, D.A., Buffington, C.A., Santiago, N.A. and Halsted, C.H. (1991) Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig. *Am. J. Physiol.*, **260**, G865–G872.

15. Halsted, C.H., Ling, E.-H., Luthi-Carter, R., Villanueva, J.A., Gardner, J.M. and Coyle, J.T. (1998) Folyl-γ-glutamate carboxypeptidase from pig jejunum. *J. Biol. Chem.*, **273**, 20417–20424.

16. Israeli, R.S., Powell, C.T., Fair, W.R. and Heston, W.D.W. (1993) Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.*, **53**, 227–230.

17. Carter, R.E., Berger, U.V., Barczak, A.K., Enna, M. and Coyle, J.T. (1998) Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. *Proc. Natl Acad. Sci. USA*, **95**, 3215–3220.

18. Luthi-Carter, R., Barczak, A.K., Speno, H. and Coyle, J.T. (1998) Molecular characterization of human brain N-acetylated α-linked acidic dipeptidase (NAALADase). *J. Pharm. Exp. Ther.*, **286**, 1020–1025.

19. Pinto, J.T., Suffoletto, B.P., Berzin, T.M., Qiao, C.H., Lin, S., Tong, W.P., May, F., Mukherjee, B. and Heston, W.D.W. (1996) Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.*, **2**, 1445–1451.

20. Luthi-Carter, R., Barczak, A.K., Speno, H. and Coyle, J.T. (1998) Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II. *Brain Res.*, **795**, 341–348.

21. O'Keefe, D.S. *et al.* (1998) Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta*, **1443**, 113–127.

22. Maraj, B.H., Leek, J.P., Karayi, M., Ali, M., Lench, N.J. and Markham, A.F. (1998) Detailed genetic mapping around a putative prostate-specific membrane antigen locus on human chromosome 11p11.2. *Cytogenet. Cell Genet.*, **81**, 3–9.

23. Rawlings, N.D. and Barrett, A.J. (1997) Structure of membrane glutamate carboxypeptidase. *Biochim. Biophys. Acta*, **1139**, 247–252.

24. Speno, H.S., Luthi-Carter, R., Macias, W.L., Valentine, S.L., Joshi, A.R.T. and Coyle, J.T. (1999) Site-directed mutagenesis of predicted active site residues in glutamate carboxypeptidase II. *Mol. Pharm.*, **55**, 179–185.

25. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.

26. Devlin, A.M., Ling, E.-H. and Halsted, C.H. (1999) Molecular studies of human intestinal folylpoly-gamma-glutamate carboxypeptidase. *Gastroenterology*, **116**, A874.

27. Kuklin, A., Munson, K., Gjerde, D., Haefele, R. and Taylor, P. (1997/1998) Detection of single-nucleotide polymorphisms with the WAVE DNA fragment analysis system. *Genet. Testing*, **1**, 201–206.

28. Liu, H., Moy, P., Kim, S., Xia, Y., Rajasekaran, A., Navarro, V., Knudsen, B. and Bander, N. (1997) Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res.*, **57**, 3629–3634.

29. Devlin, A.M., Fernando, S., Ling, E.-H., Barnetson, L., Clarke, R., Smith, A.D. and Halsted, C.H. (2000) Molecular characterization of human intestinal folylpoly-γ-glutamate carboxypeptidase (FGCP) and mutation associated with low folate status. *FASEB J.*, **14**, A202.

30. Su, S.L., Huang, I.-P., Fair, W.R., Powell, C.T. and Heston, W.D.W. (1995) Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res.*, **55**, 1441–1443.

31. Grauer, L.S., Lawler, K.D., Marignac, J.L., Kumar, A., Goel, A.S. and Wolfert, R.L. (1998) Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM′ protein in the LNCaP prostatic carcinoma cell line. *Cancer Res.*, **58**, 4787–4789.

32. Wang, T.T.Y., Chandler, C.J. and Halsted, C.H. (1986) Intracellular pteroylpoly-glutamate hydrolase from human jejunal mucosa. *J. Biol. Chem.*, **261**, 13551–13555.

33. Yao, R., Schneider, E., Ryan, T.J. and Galivan, J. (1996) Human γ-glutamyl hydrolase: cloning and characterization of the enzyme expressed in vitro. *Proc. Natl Acad. Sci. USA*, **93**, 10134–10138.

34. Bzdega, T., Turi, T., Wroblewska, B., She, D., Chung, H.S., Kim, H. and Neale, J.H. (1997) Molecular cloning of a peptidase against N-acetylaspartyl glutamate from a rat hippocampal cDNA library. *J. Neurochem.*, **69**, 2270–2277.

35. Bzdega, T., She, D., Turi, T., Wroblewska, B. and Neale, J.H. (1998) Molecular cloning of alternatively spliced variants of the peptidase against N-acetylaspartylglutamate (NAAG) from human and rat nervous systems. *Soc. Neurosci. Abstr.*, **24**, 579.

36. Finch, S., Doyle, W., Lowe, C., Bates, C.J., Prentice, A., Smithers, G. and Clarke, P.C. (1998) National diet and nutrition survey: people aged 65 years and over. Vol. 1. Report of the diet and nutrition survey. HMSO, London, UK.

37. Panel on Folate, Other B Vitamins and Choline, Food and Nutrition Board, Institute of Medicine (1998) Folate. In *Dietary Reference Intakes.* National Academy Press, Washington, WA, pp. 196–305.

38. Jacques, P.F., Bostum, A.G., Williams, R.R., Ellison, R.C., Eckfeldt, J.H., Rosenberg, I.H., Selhub, J. and Rozen, R. (1996) Relation between folate status, a common polymorphism in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*, **93**, 7–9.

39. Nygard, O., Refsum, H., Ueland, P.M. and Vollset, S.E. (1998) Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. *Am. J. Clin. Nutr.*, **67**, 263–270.

40. Bostom, A.G. and Lathrop, L. (1997) Hyperhomocysteinemia in end-stage renal disease: prevalence, etiology, and potential relationship to arteriosclerotic outcomes. *Kidney Int.*, **52**, 10–20.

41. Nygard, O., Vollset, S.E., Refsum, H., Stensvold, I., Tverdal, A., Nordrehaug, J.E., Ueland, P.M. and Kvale, G. (1995) Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. *J. Am. Med. Assoc.*, **274**, 1526–1533.

42. Frosst, P. *et al.* (1995) A candidate genetic risk factor for vascular disease: a common polymorphism in methylenetetrahydrofolate reductase. *Nature Genet.*, **10**, 111–113.

43. Sambrook, J., Fritch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

44. Ueland, P.M., Refsum, H., Stabler, S.P., Malinow, M.R., Andersson, A. and Allen, R.H. (1993) Total homocysteine in plasma or serum: methods and clinical applications. *Clin. Chem.*, **39**, 764–779.

45. Krumdieck, C.L. and Baugh, C.M. (1970) Radioactive assay of folic acid polyglutamate conjugase(s). *Anal. Biochem.*, **35**, 123–129.