American Journal of Medical Genetics 100:204–213 (2001)

Disposition of Homocysteine in Subjects Heterozygous for Homocystinuria due to Cystathionine β-Synthase Deficiency: Relationship Between Genotype and Phenotype

Anne B. Guttormsen,[1]* Per M. Ueland,[1] Warren D. Kruger,[2] Cecilia E. Kim,[2] Leiv Ose,[3] Ivar Følling,[4] and Helga Refsum[1]

[1]*LOCUS for Homocysteine and Related Vitamins, University of Bergen, Armauer Hansens Hus, Bergen, Norway*
[2]*Division of Population Science, Fox Chase Cancer Center, Philadelphia, Pennsylvania*
[3]*Lipid Clinic, Medical Department A, National Hospital, Oslo, Norway*
[4]*Section of Endocrinology, Central Hospital of Akershus, Nordbyhagen, Norway*

We have investigated 31 subjects from five unrelated families with one or more members with cystathionine β-synthase (CBS) deficiency. On the basis of their CBS genotype, the subjects were grouped as normal (n = 11) or heterozygotes (n = 20). Based on pyridoxine effect in the probands, the heterozygotes were further classified as pyridoxine-responsive (n = 9) or non-responsive (n = 11). Heterozygous subjects had normal fasting total plasma homocysteine (tHcy), but median urinary tHcy excretion rate was significantly elevated compared to healthy controls (0.39 μmol/h vs 0.24 μmol/h, $P < 0.05$). An abnormal tHcy response after methionine loading identified 73% of the pyridoxine non-responsive heterozygotes, but only 33% of the pyridoxine responsive participants. The increase in cystathionine or the change in tHcy relative to cystathionine did not improve diagnostic accuracy of the methionine loading test. After Hcy loading, the maximal increase in tHcy was significantly elevated, whereas $t_{1/2}$ was normal in heterozygotes. In conclusion, a single biochemical test cannot discriminate CBS heterozygotes from controls. Abnormal tHcy response after methionine loading was the most sensitive test. Our data suggest that the urinary tHcy excretion rate is a simple, non-invasive approach for studying mild disturbances in Hcy metabolism.

KEY WORDS: homocysteine; cystathionine β-synthase; cystathionine; genotype; phenotype

INTRODUCTION

Homocystinuria refers to rare inborn errors in homocysteine (Hcy) metabolism leading to severely elevated levels of Hcy in plasma and urine [Mudd et al., 1995; Mudd et al., 2000]. The most common cause is a deficiency of cystathionine β-synthase (CBS), the enzyme catalysing the formation of cystathionine by condensing Hcy with serine. Untreated, the afflicted subjects frequently develop skeletal abnormalities, lens dislocation, and mental retardation, and occasionally they suffer from severe thromboembolic disorders [McCully, 1969]. Hcy reducing therapy delays the development of the clinical symptoms, and markedly reduces the risk of vascular events [Wilcken and Wilcken, 1997], suggesting involvement of Hcy in the pathogenesis.

About 50% of subjects with CBS deficiency respond to pyridoxine therapy with a marked reduction in plasma total homocysteine (tHcy), whereas the remaining subjects respond poorly to such treatment. Recent studies suggest that certain mutations are associated with pyridoxine responsiveness, whereas others cause a non-responsive phenotype [Kraus et al., 1999]. However, phenotypic expression may differ markedly even in subjects with the same genotype [Dawson et al., 1996], which may suggest that other genetic and/or environmental factors are involved.

Grant sponsor: Norwegian Council on Cardiovascular Disease; Grant sponsor: Norwegian Research Council; Grant sponsor: EU Commission Demonstration Project; Grant number: BMH4-98-3549.

*Correspondence to: Dr. Anne B. Guttormsen, Department of Pharmacology, University of Bergen, Armauer Hansens hus, N-5021 Bergen, Norway. E-mail: Anne.Guttormsen@farm.uib.no

Received 23 June 2000; Accepted 17 January 2001

Published online 23 March 2001

CBS deficiency is an autosomal recessive trait. A study of obligate heterozygotes indicates that they do not have increased vascular risk [Mudd et al., 1981], but others [Rubba et al., 1990; Swift and Morrell, 1982] have contested this. There are indications that CBS heterozygosity may interact with other risk factors [Yap et al., 1999], in line with observations in homozygous patients [Kluijtmans et al., 1998; Mandel et al., 1996]. Hence, heterozygosity may be a weak risk factor for disease in a subgroup of susceptible patients.

Although the enzyme activity is reduced, most heterozygous subjects have normal fasting tHcy levels, but they frequently respond to a methionine loading with an abnormal increase in mixed disulphide [Sardharwalla et al., 1974] or tHcy [Tsai et al., 1996]. The clinical significance of this finding is uncertain. Although increased post methionine load (PML) tHcy level is a risk factor for vascular disease [Graham et al., 1997] and neural tube defects [Steegers-Theunissen et al., 1994], heterozygosity for CBS deficiency is uncommon in these conditions [Kluijtmans et al., 1996; Morrison et al., 1998; Ramsbottom et al., 1997]. This may be related to the fact that the prevalence of CBS heterozygosity is low in the general population, or that heterozygosity is not a predisposing condition. A third possibility is that only the subset of heterozygous subjects with an abnormal Hcy metabolism is at increased risk of disease [Tsai et al., 1999].

There are sparse data on the relation between genotype and phenotype in heterozygous CBS-deficient subjects [Dawson et al., 1996; Sperandeo et al., 1996; Tsai et al., 1996]. Moreover, previous studies have only investigated fasting and PML tHcy levels. In the present study, we have analysed plasma tHcy and cystathionine before and after methionine loading in 31 relatives from five unrelated families with at least one subject with genetically verified CBS deficiency. In a subset, we investigated the disposition of Hcy by measuring tHcy in plasma and urine before and following a Hcy loading test.

SUBJECTS AND METHODS

Subjects

Subjects with homocystinuria due to CBS deficiency were recruited from medical practices and hospitals in Southern Norway. The diagnosis of homocystinuria was originally based on clinical symptoms and high levels of tHcy in plasma or urine, and recently the diagnosis was confirmed by genetic analysis [Kim et al., 1997].

Invitation to participate in the study was sent to eight families. In the present study, we have restricted the analysis to those families where one or more of the relatives agreed to undergo a methionine loading test. Hence, the study population included 31 subjects, 15 women and 16 men, aged 15–67 yr (median 48 yr) from five unrelated families (coded A, B, E, F, and G). Details on demographics, genetics, and treatment are given in Table I. The Regional Ethics Committee in Health Region III approved the study.

Protocol

From all subjects we collected a fasting blood sample for the determination of the serum/plasma levels of tHcy, methionine, cystathionine, vitamins, creatinine, and for CBS and methylenetetrahydrofolate (MTHFR) genotyping. A morning urine sample was obtained from 15 subjects. All subjects underwent a methionine loading test.

The Hcy loading test was performed in 10 of the heterozygous CBS participants [Guttormsen et al., 1993].

The methionine and Hcy loading tests were performed on separate days, at least two weeks apart, to avoid carry-over effects.

The methionine loading test. The administration of methionine was routinely performed in the morning after an overnight fast. Briefly, methionine powder, 100 mg/kg (671 μmol/kg) in orange juice was ingested within 30 sec [Fowler et al., 1971; Sardharwalla et al., 1974]. Blood samples were collected immediately before and 6 hr after administration of methionine.

The Hcy loading test. Immediately before administration, a solution containing L-homocysteine was prepared by treating L-homocysteine thiolactone, 10 mg/kg (65 μmol/kg) with NaOH, followed by neutralisation with HCl, and dilution with water and apple juice [Guttormsen et al., 1993]. The solution was ingested over a period of 1–2 min. From each participant, blood samples were collected immediately before Hcy ingestion, and after 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 hr. Urine was collected in fractions for 24 hr in addition to the morning urine sample.

Laboratory Procedures

Blood for mutation analysis was collected into evacuated tubes containing EDTA and then stored at -80°C. Plasma used for determination of tHcy, methionine, methylmalonic acid, and cystathionine was prepared from blood collected into an evacuated EDTA tube. The blood was centrifuged immediately, or stored on ice for up to 1 hr before centrifugation. The plasma fraction was transferred to new vials that were stored at -20°C until analysis. Serum was prepared by collecting blood into an evacuated tube without anticoagulant, the whole blood was allowed to coagulate for 0.5–1 hr, and then serum was prepared by centrifugation. Aliquots for determination of serum levels of creatinine, folate, and cobalamin were analysed within 1–2 d.

The analyses of mutations and polymorphisms of the CBS and MTHFR genes were performed in all the participating subjects as described [Kim et al., 1997; Ulvik et al., 1997]. The results from some of the subjects have been published previously [Kim et al., 1997], and the complete data set is presented in Table I.

The concentration of tHcy in plasma and urine was determined by a modification [Fiskerstrand et al., 1993] of an automated procedure developed for the determination of tHcy in plasma [Refsum et al., 1989]. Plasma

TABLE I. Demographics, Genetics and Therapy

Family	Subject	Age	Sex	Relation to proband[a]	CBS mutation	Pyr. resp.[b]	68-bp ins.[c]	677C→T-MTHFR[d]	Therapy[e]
Group I, homozygotes									
A	1	24	M		797G→A/797G→A	+	+ /+	CT	B6, FA
B	2	26	M		797G→A/797G→A	+	+ /+	CC	B6, FA
B	3	21	M		797G→A/797G→A	+	+ /+	CT	B6
E	6	19	F		785C→T/785C→T	–	– /–	CC	B6, FA, Betaine
F	7	15	M		919G→A/959T→C	–	–/+	CT	B6, FA, Betaine
G	8	20	F		919G→A/959T→C	–	–/+	CT	B6, FA, Betaine
Group II, heterozygotes									
A	10	46	F	Mo	797G→A/wt	+	–/+	CT	
A	11	48	M	Fa	797G→A/wt	+	–/+	CT	
A	20	21	M	Br	797G→A/wt	+	–/+	TT	
A	21	67	F	Grm	797G→A/wt	+	–/+	CT	
A	22	62	F	Pr	797G→A/wt	+	–/+	CC	
B	12	46	F	Mo	797G→A/wt	+	–/+	CT	
B	13	49	M	Fa	797G→A/wt	+	–/+	CT	
B	23	23	M	Br	797G→A/wt	+	–/+	CC	
B	24	15	F	Si	797G→A/wt	+			
E	14	45	F	Mo	785C→T/wt	–	–/–	CT	
E	15	49	M	Fa	785C→T/wt	–	–/–	CC	
E	25	16	M	Br	785C→T/wt	–	–/–	CT	
F	16	52	F	Mo	919G→A/wt	–	–/–	CC	FA
F	26	24	F	Si	919G→A/wt	–	–/–	CT	
F	29	49	M	Un	919G→A/wt	–	–/–	CT	
G	18	52	M	Fa	919G→A/wt	–	–/–	CT	Multivitamin
F	17	51	M	Fa	959T→C/wt	–	–/+	TT	FA
F	27	28	F	Si	959T→C/wt	–	–/+	TT	
F	30	53	F	Au	959T→C/wt	–	–/+	CC	
G	19	53	F	Mo	959T→C/wt	–	–/+	CC	Multivitamin
Group III, no mutation									
A	31	35	F	Au	wt/wt		–/–	CT	
A	32	51	F	Pr	wt/wt		–/–	CC	
A	33	57	M	Pr	wt/wt		–/–	CC	
A	34	61	F	Pr	wt/wt		–/–	CC	
F	36	48	M	Un	wt/wt		–/–	CC	
F	37	46	M	Un	wt/wt		–/–	CT	
F	38	27	M	Un	wt/wt		–/–	CC	
F	39	44	M	Un	wt/wt		–/–	CT	
F	40	54	M	Un	wt/wt		–/–	CT	
F	41	37	M	Un	wt/wt		–/–	CC	
F	42	48	F	Au	wt/wt		–/–	CT	

[a]Mo, mother; Fa, father; Br, Brother; Grm, grandmother; Pr, peripheral relative; Si, sister; Un, uncle; Au, Aunt.
[b]Pyridoxine reponsive.
[c]The 68-base pair insertion on exon 8 of the CBS gene.
[d]The 677C→T polymorphism in the methylenetetrahydrofolate gene; CC, homozygous not mutated; CT, heterozygous mutated; TT, homozygous mutated.
[e]FA, folic acid.

methionine was determined in plasma samples with an assay based on derivatisation with o-phthaldialdehyde and fluorescence detection [Krishnamurti et al., 1984]. Plasma levels of methylmalonic acid and cystathionine were measured using a slight modification of a GC-MS method based on ethylchloroformate derivatisation [Husek, 1998]. Serum cobalamin was determined with a microparticle enzyme intrinsic factor assay run on an IMx system from Abbott (Abbott Park, Il), and serum folate was assayed using the Quantaphase folate radioassay produced by Bio Rad (Hercules, CA).

Reference Ranges

For creatinine, methylmalonic acid, and the vitamins, the normal ranges as defined in the university hospital clinical chemistry laboratory were used. The upper normal limits for serum methylmalonic acid and creatinine are 0.26 μmol/L and 120 μmol/L, respectively. The lower normal limit for serum cobalamin is 150 pmol/L, and for serum folate 4.5 nmol/L. The normal range for methionine is defined as 10–40 μmol/L [Blom et al., 1992], and the normal range for tHcy is defined as 5–15 μmol/L [Refsum et al., 1998]. A methionine level of 40 μmol/L or tHcy level of 15 μmol/L corresponds to the 90–95 percentile in blood samples collected from adult study populations [Nygård et al., 1998; Graham et al., 1997]. The normal urinary excretion rate in healthy adults was calculated from morning urine samples collected from 93 subjects (41% females), aged 20–64 yr (Table II). The reference ranges (defined as the 5^{th} and 95^{th} percentiles) for cystathionine before and after methionine loading, and tHcy after methionine loading were established in a

subset of the control population of the European Concerted Action (COMAC) study [Graham et al., 1997], and included 97 subjects (48% female) with a median age of 45 yr (range 20–59 yr).

The reference ranges for the response after the Hcy load (Table IV) are derived from 19 healthy subjects, aged 19–58 yr (42% female), recruited from the laboratory staff.

Calculation of Kinetic Parameters

The elimination of tHcy after oral intake obeys first order kinetics and is consistent with a one-compartment model. For simplicity, and for comparison with our previous studies [Guttormsen et al., 1993; Guttormsen et al., 1996; Guttormsen et al., 1997], the elimination rate constant (k_e) and $t_{1/2}$ were calculated by linear regression of the terminal linear part (from 2–6 hr) of the log-transformed concentration versus the time curve. The kinetic parameters were calculated using KaleidaGraph TM, version 2.1.3 for Macintosh (Synergy Software, Reading, PA). The formulas used for the calculations are given elsewhere [Guttormsen et al., 1993].

Statistical Methods

The results are given as median or mean and SD or range. Unpaired values were evaluated by the Mann-Whitney U test. Simple correlation was estimated by the Spearman rank correlation test. The significance level was set to 5%.

To describe the diagnostic accuracy of the tests, we used Receiver Operating Characteristic (ROC) plots [Hanley and McNeil, 1982; Metz, 1978; Zweig and Campbell, 1993]. The ROC plots were constructed by using the software ASTUTE for PC (DDU Software, Leeds LS2 9JT, UK).

RESULTS

Genotyping

Twenty participants were heterozygous, and 11 had no CBS mutation. Based on the response to pyridoxine therapy in the probands, we further classified a subject as a responsive or non-responsive phenotype (Table I). The three responsive probands, belonging to families A and B, were homozygous for the 797G→A (R266K) mutation. The three non-responsive probands had two different genotypes; subject E6 was homozygous for the 785C→T (T262M) mutation, whereas F7 and G8 were compound heterozygotes, with 919G→A (G307S) mutation in one allele and the 959T→C (V320A) mutation in the other allele. Among subjects identified as being heterozygous, nine carried the pyridoxine-responsive 797G→A mutation. Among the remaining 11 heterozygous subjects with a non-responsive mutation, three had the 785C→T mutation, four had the 919G→A, and four carried the 959T→C mutation.

Among the heterozygous CBS deficient participants, one was homozygous and 11 were heterozygous for the 68 bp insertion (Table I). Based on the results in the probands, it seems that the 68 bp insertion occurs in cis with the 797G→A and 959T→C mutations in these families. Three of the subjects had the MTHFR TT genotype. Neither the CBS 68 bp insertion nor the MTHFR genotype was associated with abnormal tHcy or cystathionine levels before or after methionine or Hcy loading.

Fasting Vitamin and Metabolite Levels

One subject (A33) had cobalamin deficiency (plasma tHcy 22.5 μmol/L; serum cobalamin 104 pmol/L; methylmalonic acid 0.45 μmol/L). The remaining subjects had normal serum concentrations of cobalamin, and creatinine was within the reference limits. Serum folate was < 4.5 nmol/L in three subjects without a CBS mutation (F36, F38, F39) (data not shown).

Levels and the distributions of fasting tHcy, were similar to those observed in healthy adults, and did not differ between those with and those without a mutated allele in CBS. Fasting hyperhomocysteinemia (> 15 μmol/L) was only observed in two subjects, one with heterozygosity for the 785C→T mutation (E15), and one with wild type CBS but with additional cobalamin deficiency (A33). Hence, heterozygosity for CBS does not seem to influence the fasting tHcy in these families (Table II).

Cystathionine tended to be lower in the CBS heterozygotes ($P = 0.10$), but a low cystathionine level was infrequent.

Methionine was normal in the pyridoxine non-responsive heterozygotes, and low in the pyridoxine responsive CBS heterozygotes. This was caused by low plasma levels in the members of family B. Moreover, a high methionine level was observed in two subjects, E14 (heterozygote) and A33 (wild type).

Urinary tHcy Excretion Rate

The urinary excretion rate of tHcy was determined in a urine sample collected in the morning. In a normal adult population with a median plasma tHcy level of 8.4 μmol/L, the median, 5^{th} and 95^{th} percentile of the urinary excretion rate were 0.24, 0.09, and 0.48 μmol/h, respectively. The excretion rate correlated with plasma tHcy ($Rs = 0.30$, $P = 0.01$).

In the heterozygotes, median urinary tHcy excretion rate was 0.39 μmol/h, significantly higher ($P < 0.001$) than in controls. Five of the 15 heterozygotes had an excretion rate above the defined upper limit of 0.48 μmol/h (Table II). The trend towards an elevated Hcy excretion rate was observed both in the pyridoxine responsive (median 0.41 μmol/h) and in the pyridoxine non-responsive subjects (median 0.37 μmol/h).

Hcy Response to Methionine Loading

The median increase in tHcy and the ratio between post and pre tHcy levels were 31.6 μmol/L and 4.1, respectively, i.e., markedly higher than the median values observed in controls (18.4 μmol/L and 3.0, respectively). Notably, this was mainly related to the abnormal tHcy response in the CBS heterozygotes.

TABLE II. Fasting Plasma Metabolite Levels and tHcy Urinary Excretion Rate*

Family	Subject	Plasma (μmol/L) tHcy	Methionine	Cystathionine	Urinary tHcy excretion μmol/h
Heterozygotes, pyridoxine responsive					
A	10	6.9	26.1	0.093	**0.92**
A	11	8.9	32.2	0.202	0.47
A	20	12.4	29.9	0.069	0.23
A	21	7.9	19.5	0.073	
A	22	12.4	28.6	0.353	
B	12	6.9	16.5	**0.064**	0.41
B	13	12.7	14.8	0.080	**0.59**
B	23	11.9	18.5	0.071	0.26
B	24	7.5	16.5	0.082	0.31
Median		*8.9*	*19.5*	*0.080*	*0.41*
Heterozygotes, pyridoxine non-responsive					
E	14	12.3	**42.3**	0.093	**0.78**
E	15	**16.7**	28.3	0.159	**0.63**
E	25	10.5	33.5	0.104	
F	16	5.7	19.9	**0.042**	0.13
F	26	7.5	39.3	0.143	
F	29	9.5	24.5	0.098	0.39
G	18	9.7	34.8	0.172	0.34
F	17	9.0	25.2	0.069	0.30
F	27	8.7	23.6	0.098	
F	30	7.5	20.7	0.074	0.16
G	19	9.8	39.8	0.160	**1.16**
Median		*9.5*	*28.3*	*0.098*	*0.37*
Subjects without mutation					
A	31	5.7	23.8	**0.063**	
A	32	11.6	26.4	0.096	
A	33	**22.5**	**48.4**	0.277	
A	34	9.9	35.5	0.157	
F	36	8.2	33.8	0.184	
F	37	5.9	29.8	0.179	
F	38	10.8	26.7	0.207	
F	39	11.2	32.1	0.136	
F	40	9.7	25.9	0.216	
F	41	7.1	29.3	0.095	
F	42	6.2	30.9	0.097	
Median		*9.7*	*29.8*	*0.157*	
Reference limits		5–15	10–40	0.065–0.190	0.09–0.48

*Abnormal values related to CBS function are marked in bold.

However, four of the subjects with wild type CBS showed an abnormal increase in tHcy. Among these one (A33) had cobalamin deficiency and three (F36, F38, F39) had low serum folate levels. Only one (F38) had abnormally high post/pre tHcy ratio.

Among the 20 heterozygous participants, 13 (65%) had an abnormal increase in tHcy and/or elevated post/pre tHcy ratio. By also taking the test results from the ∂tHcy/∂cystat ratio, the tHcy urinary excretion rate and the C_{max} after Hcy loading into account 17 (85%) of the heterozygotes are identified (Tables II, III and IV).

A larger portion of the pyridoxine non-responsive subjects tended to have an abnormal PML tHcy response as compared with the pyridoxine responsive subjects (Table III). This trend was most pronounced for the ratio between post and pre tHcy levels. This ratio identified 73% (8/11) of the pyridoxine non-responsive subjects, and three out of nine subjects (33 %) with the pyridoxine responsive phenotype.

Cystathionine Response to Methionine Loading

The fasting median cystathionine was 0.093 μmol/L in heterozygotes compared to 0.113 μmol/L in controls ($P = 0.08$). Six hours after methionine loading, the median increase in cystathionine in heterzygotes was 0.693 μmol/L, which is identical to the increase observed in the control population ($P = 0.67$). We also investigated the ratio between the increase in tHcy and increase in cystathionine. This ratio differed considerably among the participants. In subjects with normal CBS, the ratio was significantly lower than in the control population ($P = 0.01$), while it was significantly higher in the heterozygotes ($P < 0.001$) (Table III).

TABLE III. Response to Methionine Loading*

Family	Subject	∂tHcy[a]	Post tHcy/pre tHcy[b]	∂Cysta[c]	Post cysta/pre cysta[d]	∂tHcy/∂Cysta[e]
Heterozgotes, pyridoxine responsive						
A	10	**48.4**	**8.0**	0.655	8.0	**73.9**
A	11	**28.6**	**5.3**	0.461	**3.3**	**83.6**
A	20	30.9	3.5	0.402	6.8	**76.9**
A	21	22.1	3.8	**0.262**	4.6	**84.3**
A	22	**38.0**	4.1	3.731	11.6	10.2
B	12	**61.4**	**9.9**	**0.241**	4.8	**254.6**
B	13	15.9	2.3	0.731	10.1	21.7
B	23	21.4	2.8	0.541	8.6	39.7
B	24	17.0	3.3	0.914	12.1	18.6
Median		*30.9*	*3.8*	*0.541*	*8.0*	*73.9*
Heterozygotes, pyridoxine non-responsive						
E	14	**64.6**	**6.3**	1.011	11.9	63.9
E	15	**60.2**	**4.6**	0.837	6.3	**71.9**
E	25	**32.3**	4.1	0.616	6.9	52.4
F	16	29.3	**6.1**	0.532	13.7	55.1
F	26	**55.7**	**8.4**	1.199	9.4	46.5
F	29	24.6	3.6	0.563	6.7	43.6
G	18	27.7	3.9	0.883	6.1	31.4
F	17	**43.5**	**5.8**	1.318	20.1	33.0
F	27	**36.3**	**5.2**	0.931	10.5	39.0
F	30	**31.6**	**5.2**	0.902	13.2	35.0
G	19	**35.3**	**4.6**	0.373	**3.3**	**94.6**
Median		*35.3*	*5.2*	*0.883*	*9.4*	*46.5*
Subjects without mutation						
A	31	16.9	4.0	0.833	14.2	20.4
A	32	26.0	3.2	0.876	10.1	29.7
A	33	**36.1**	2.6	0.780	3.8	46.2
A	34	17.8	2.8	0.788	6.0	22.6
F	36	16.9	3.1	5.509	30.9	3.1
F	37	18.1	4.1	1.490	9.3	12.1
F	38	**38.4**	**4.6**	5.188	26.1	7.4
F	39	**35.4**	4.2	1.335	10.8	26.5
F	40	**31.9**	4.3	9.273	43.9	3.4
F	41	20.4	3.9	1.408	15.8	14.5
F	42	16.4	3.6	2.550	27.3	6.5
Median		*20.4*	*3.9*	*1.408*	*14.2*	*14.5*
Reference levels		12–31	2.3–4.4	0.300–2.200	3.5–18	8–70

*Abnormal values related to CBS function are marked in bold.
[a]The increase in tHcy, μmol/L.
[b]The ratio between post and pre tHcy.
[c]The increase in cystathionine, μmol/L.
[d]The ratio between post and pre cystathionine.
[e]The ratio between the increase in tHcy and the increase in cystathionine.

Thus, the cystathionine levels in these subjects seemed to be more related to family than CBS genotype (see below).

The Hcy Loading

Within one hr after Hcy intake, tHcy increased to a peak value, C_{max} (Fig. 1). In the heterozygous subjects, the average (± SD) C_{max} was 77.3 ± 18.8 μmol/L and significantly higher than the C_{max} of 61.5 ± 12.1 μmol/L observed in healthy subjects ($P = 0.02$). Seven out of the 10 heterozygous subjects had a $C_{max} \geq$ the 90th percentile of the controls. The $t_{1/2}$ was slightly lower than in the control population, and none of the heterozygous subjects had $t_{1/2}$ above the 90th percentile of the controls. The area under the curve was non-significantly higher than in the controls.

From nine heterozygous subjects, we collected urine during the Hcy loading. About 3% of the administered dose were excreted unchanged in the urine. This fraction showed a strong association to the C_{max} of the tHcy increase ($Rs = 0.75$, $P = 0.03$).

The methionine response to Hcy loading was only marginally higher than observed in healthy subjects (Table IV).

ROC Analyses

A large area under the ROC curve indicates that a variable has a good ability to discriminate between heterozygous subjects and subjects without a mutation. A test with an area that is not significantly greater than 0.5 lacks discriminatory power. Except fasting tHcy and fasting cystathionine, all the other measures (Fig. 2) discriminated significantly between the two groups of subjects. Among the tests based on a single measurement, urinary tHcy excretion rate gave the largest area under the ROC curve.

TABLE IV. Response to Hcy Loading*

Family	Subject	C_{max}tHcy[a]	C_{max}methionine[b]	AUC[c]	$t_{1/2}$[d]	Urinary tHcy[e]
Heterozygotes, pyridoxine responsive						
A	10	**84.6**	10.0	314	2.7	3.1
A	11	**114.2**	12.4	553	2.9	4.2
B	12	49.1	8.9	**468**	3.5	2.0
B	13	52.9	20.5	418	3.6	2.5
B	23	68.3	14.0	276	3.3	2.1
B	24	**78.0**	12.8	403	3.0	3.4
Median		*73.2*	*12.6*	*411*	*3.2*	*2.8*
Heterozygotes,pyridoxine non-responsive						
E	14	73.8	12.3	448	3.4	3.5
E	15	**89.4**	16.9	580	2.8	2.7
F	16	73.8	19.8	433	3.2	
F	17	**88.6**	12.5	655	3.6	3.7
Median		*81.2*	*14.7*	*514*	*3.3*	*3.5*
Reference limits		47.3–73.8	8.7–22.4	360–460	2.9–4.7	

*Abnormal values related to CBS function are marked in bold.
[a]The maximal increase in tHcy, μmol/L.
[b]The maximal increase in methionine, μmol/L.
[c]The area under the curve, μmol/L.
[d]Elimination half-life, hr.
[e]The percentage of the given dose of Hcy excreted in the urine within 24 hr after Hcy loading, %.

Differences Between Families

Family A (pyridoxine responsive) and family F (pyridoxine non-responsive), comprised nine and 13 family members, respectively. Neither fasting tHcy, fasting cystathionine, nor the post/pre tHcy ratio after methionine loading was significantly different compared to the controls, and these parameters were nearly identical between the two families, independent of CBS carrier status. A stronger PML increase in cystathionine was observed in family F, and in particular in the members without a mutated allele (median 2.55 μmol/L). Notably, five out of seven participants in family F without a mutation had serum folate ≤ 5 nmol/L. Therefore, folate status rather than the CBS genotype may explain the cystathionine response in these subjects [Stabler et al., 1993].

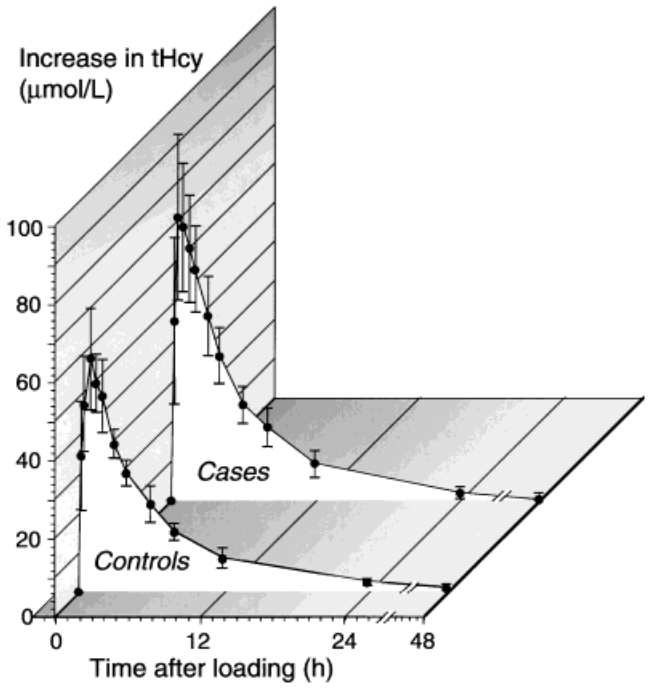

Fig. 1. Plasma tHcy elimination curves after oral Hcy loading (65 μmol/kg bw) in 10 participants heterozygous for CBS deficiency (cases), and in 19 healthy subjects (controls). The mean tHcy plasma concentrations with standard deviations (vertical bars) are plotted versus time.

DISCUSSION

In order to identify markers that are characteristic for CBS carrier status, we investigated fasting levels of tHcy and cystathionine, the tHcy urinary excretion rate and the disposition of Hcy and related metabolites after methionine and Hcy loadings. Among these, the ratio between post and pre tHcy in response to methionine loading was the test with highest diagnostic sensitivity and specificity, and identified 73% of the pyridoxine non-responsive heterozygotes. By combining the ∂tHcy/∂cysta ratio, the tHcy urinary excretion rate, and the C_{max} after Hcy loading, 17 out of 20 heterozygotes were identified. Notably, Hcy loading identified only one extra individual compared to methionine loading, suggesting that this labour intensive test should not be a part of the battery of tests performed to identify CBS carrier status. The urinary tHcy excretion rate was also significantly elevated in CBS mutation carriers, and may be a simple and practical test in the clinical setting.

There was no consistent relation between the 68 bp insertion in the CBS gene or the 677C→T polymorphism in the MTHFR gene and the tHcy response to methionine loading (Table I).

According to the literature, the two most common mutations in the CBS gene are the pyridoxine responsive 833T→C (I278T) and the pyridoxine non-responsive 919G→A (G307S) [Kraus et al., 1999]. Both these

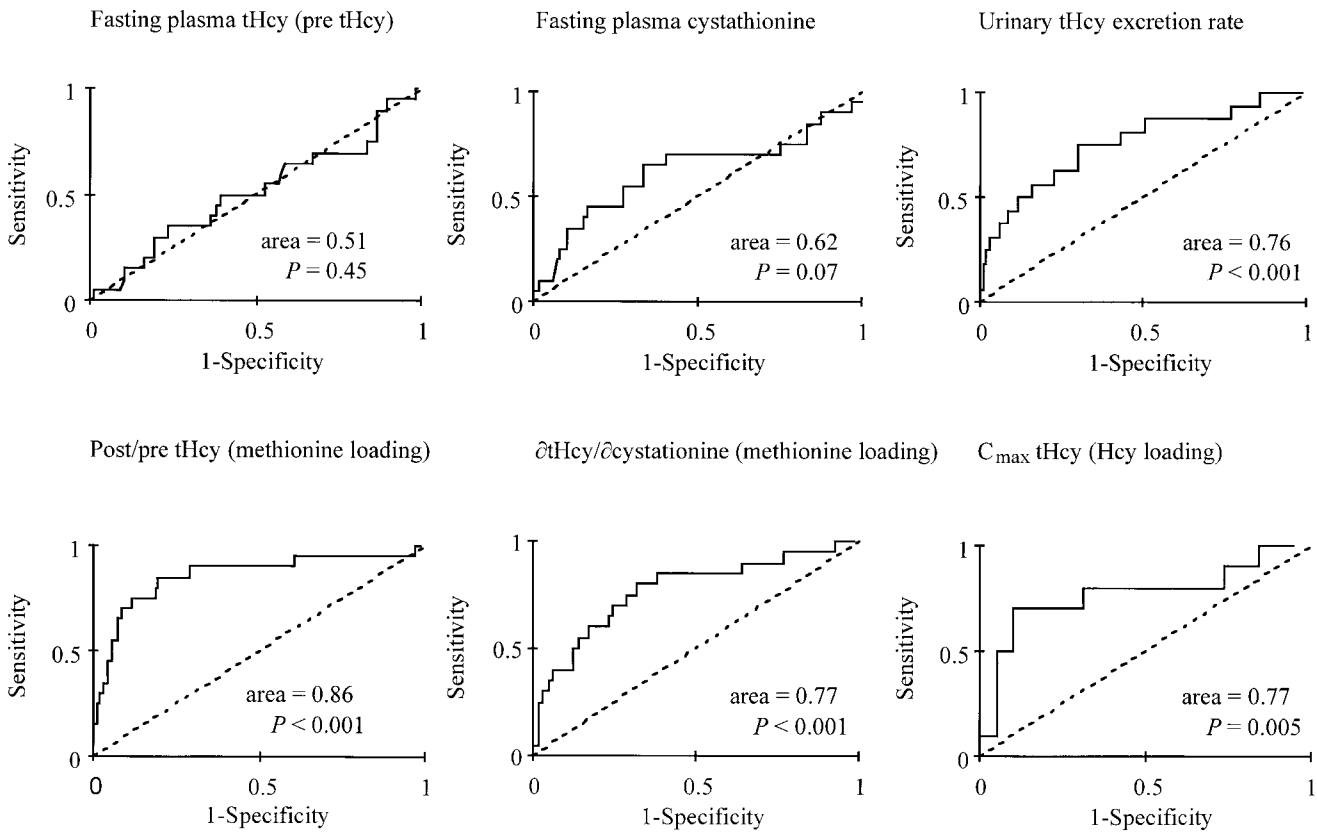

Fig. 2. Discrimination by ROC plots. The upper panel shows that among the tests based on a single measurement, the urinary tHcy excretion rate had a better diagnostic performance than both fasting tHcy and fasting cystathionine. The lower panel demonstrates that the post/pre tHcy ratio after a methionine loading has a better diagnostic performance than the ratio between the increase in tHcy and the increase in cystathionine and the C_{max} after Hcy loading.

mutations have been identified in Norwegian patients (Table I) [Kim et al., 1997]. However, the recently discovered 797G→A (R266K) mutation [Kim et al., 1997] seems to be the most common cause of homocystinuria in Norway. In a yeast model [Kruger and Cox, 1994], this allele has a normal phenotype when exposed to high concentrations of pyridoxine, but when pyridoxine concentration is low, the mutation exhibit a severely abnormal phenotype. This mirrors the genotype–phenotype relation seen in humans that shows that the tHcy level is highly dependent on the pyridoxal 5-phosphate status of the patient [Kim et al., 1997].

The urinary excretion rate of tHcy is proportional to the plasma tHcy concentration [Guttormsen et al., 1996], and CBS deficiency was initially called homocystinuria due to the finding of an extremely elevated Hcy level in the urine [Mudd et al., 1964; Mudd et al., 2000]. When investigating 12 obligate heterozygotes and 12 normal subjects, Sardharwalla et al. [1974] found that preload urine samples did not reveal any differences in sulphur amino acid concentration between cases and controls. In the present study, the plasma tHcy level was similar for heterozygotes and controls, but despite this, the preload urinary tHcy excretion rate was significantly higher in the heterozygotes. Hence, our data suggest that measurement of the urinary tHcy excretion rate should be further evaluated as a sensitive test to detect mild disturbances in Hcy metabolism not revealed by measurement of fasting tHcy levels.

The biochemical conversion of Hcy to cysteine, denoted transsulfuration, depends on two consecutive, pyridoxal 5-phosphate-dependent reactions. Oral methionine is believed to stress the transsulfuration pathway, and has been used to identify CBS carrier status [Brenton et al., 1965; Fowler et al., 1971; Sardharwalla et al., 1974]. However, recent studies have revealed that PML tHcy response is dependent both on pyridoxine [Ubbink et al., 1996] and on MTHFR and folate status [Cattaneo et al., 1999; Nelen et al., 1998]. Hence, an abnormal response to methionine loading may be a common but not a very specific feature. In the present study, the PML tHcy response was significantly higher in the heterozygous subjects than in the controls, but it did not completely discriminate between these two groups (Fig. 2). Previous studies have frequently used age and sex specific limits for the response to methionine loading [Andersson et al., 1992; Silberberg et al., 1997]. However, using age and sex specific limits based on the total COMAC control population (n = 800) [Graham et al., 1997; Refsum et al., 1998], did not markedly alter our results.

We found that three out of four relatives with wild type CBS and abnormal PML tHcy had low serum folate, < 4.5 nmol/L, in line with the observations done by Nelen and co-authors [Nelen et al., 1998].

In a small study performed by Stabler et al. [1993], it was observed that serum cystathionine did not distinguish between CBS heterozygosity and wild type. They suggested that the ratio between the increase in cystathionine and increase in tHcy after a methionine loading might discriminate better than the increase in plasma tHcy. However, our results do not support this possibility.

After Hcy loading, the plasma tHcy elimination kinetics was the same in the heterozygous subjects and in controls [Guttormsen et al., 1993] (Table IV). However, C_{max} was significantly higher [Guttormsen et al., 1993] (Fig. 1). One possible explanation could be a difference in distribution volume. A more likely cause, is that the defect transsulfuration pathway impairs the first pass metabolism of Hcy following its absorption.

Our results show that unequivocal identification of CBS carrier status requires DNA analysis. Still, CBS heterozygosity is associated with abnormal Hcy metabolism in a substantial proportion of the subjects, but it remains uncertain whether this conveys an increased risk of cardiovascular disease or other clinical conditions associated with hyperhomocysteinemia. We recommend that future studies on the relation between CBS heterozygosity and various clinical endpoints should include genotyping as well as assessment of in vivo CBS function. Our data suggest that such testing should include measurement of tHcy in plasma before and after methionine loading as well as urinary tHcy excretion.

ACKNOWLEDGMENTS

We thank cand. scient. Arve Ulvik for performing the MTHFR analyses, and Elfrid Blomdal, Ove Årseth, Ove Netland, Jennifer Gjerde, and Wenche Breyholtz for excellent technical assistance. We also thank Ricky Sheahan, presently at University of Texas Medical Branch at Galveston, Texas, professor Ian Graham, Department of Cardiology, Adelaide-Meath Hospital, Incorporating the National Children's Hospital, Tallaght, Dublin, Ireland, and professor Lars Brattström, County Hospital, Kalmar, Sweden for providing relevant blood samples from the COMAC study population.

REFERENCES

Andersson A, Brattstr&oumlm L, Israelsson B, Isaksson A, Hamfelt A, Hultberg B. 1992. Plasma homocysteine before and after methionine loading with regard to age, gender, and menopausal status. Eur J Clin Invest 22:79–87.

Blom HJ, Davidson AJ, Finkelstein JD, Luder AS, Bernardini I, Martin JJ, Tangerman A, Trijbels JM, Mudd SH, Goodman SI, et al. 1992. Persistent hypermethioninaemia with dominant inheritance. J Inherit Metab Dis 15:188–197.

Brenton DP, Cusworth DC, Gaull GE. 1965. Homocystinuria: metabolic studies on 3 patients. J Pediatr 67:58–68.

Cattaneo M, Lombardi R, Lecchi A, Zighetti ML. 1999. Is the oral methionine loading test insensitive to the remethylation pathway of homocysteine? Blood 93:1118–1120.

Dawson PA, Cochran DA, Emmerson BT, Kraus JP, Dudman NP, Gordon RB. 1996. Variable hyperhomocysteinaemia phenotype in heterozygotes for the Gly307Ser mutation in cystathionine beta-synthase. Aust N Z J Med 26:180–185.

Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. 1993. Homocysteine and other thiols in plasma and urine: automated determination and sample stability. Clin Chem 39:263–271.

Fowler B, Sardharwalla IB, Robins AJ. 1971. The detection of heterozygotes for homocystinuria by oral loading with L-methionine. Biochem J 122:23–24.

Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, Palma-Reis RJ, Boers GH, Sheahan RG, Israelsson B, Uiterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, de Valk HW, et al. 1997. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 277:1775–1781.

Guttormsen AB, Mansoor MA, Fiskerstrand T, Ueland PM, Refsum H. 1993. Kinetics of plasma homocysteine in healthy human subjects after peroral homocysteine loading. Clin Chem 39:1390–1397.

Guttormsen AB, Schneede J, Ueland PM, Refsum H. 1996. Kinetics of total plasma homocysteine in subjects with hyperhomocysteinemia due to folate or cobalamin deficiency. Am J Clin Nutr 63:194–202.

Guttormsen AB, Ueland PM, Svarstad E, Refsum H. 1997. Kinetic basis of hyperhomocysteinemia in patients with chronic renal failure. Kidney Int 52:495–502.

Hanley JA, McNeil BJ. 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143:29–36.

Husek P. 1998. Chloroformates in gas chromatography as general purpose derivatizing agents. J Chromatogr B Biomed Sci Appl 717:57–91.

Kim CE, Gallagher PM, Guttormsen AB, Refsum H, Ueland PM, Ose L, Folling I, Whitehead AS, Tsai MY, Kruger WD. 1997. Functional modeling of vitamin responsiveness in yeast: a common pyridoxine-responsive cystathionine beta-synthase mutation in homocystinuria. Hum Mol Genet 6:2213–2221.

Kluijtmans LA, van den Heuvel LP, Boers GH, Frosst P, Stevens EM, van Oost BA, den Heijer M, Trijbels FJ, Rozen R, Blom HJ. 1996. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 58:35–41.

Kluijtmans LA, Boers GH, Verbruggen B, Trijbels FJ, Novakova IR, Blom HJ. 1998. Homozygous cystathionine beta-synthase deficiency, combined with factor V Leiden or thermolabile methylenetetrahydrofolate reductase in the risk of venous thrombosis. Blood 91:2015–2018.

Kraus JP, Janosik M, Kozich V, Mandell R, Shih V, Sperandeo MP, Sebastio G, de Franchis R, Andria G, Kluijtmans LA, Blom H, Boers GH, Gordon RB, Kamoun P, Tsai MY, Kruger WD, Koch HG, Ohura T, Gaustadnes M. 1999. Cystathionine beta-synthase mutations in homocystinuria. Hum Mutat 13:362–375.

Krishnamurti CR, Heindze AM, Galzy G. 1984. Application of reversed-phase high-performance liquid chromatography using pre-column derivatization with o-phthaldialdehyde for the quantitative analysis of amino acids in adult and fetal sheep plasma, animal feeds and tissues. J Chromatogr 315:321–331.

Kruger WD, Cox DR. 1994. A yeast system for expression of human cystathionine beta-synthase: structural and functional conservation of the human and yeast genes. Proc Natl Acad Sci U S A 91:6614–6618.

Mandel H, Brenner B, Berant M, Rosenberg N, Lanir N, Jakobs C, Fowler B, Seligsohn U. 1996. Coexistence of hereditary homocystinuria and factor V Leiden–effect on thrombosis. N Engl J Med 334:763–768.

McCully KS. 1969. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 56:111–128.

Metz CE. 1978. Basic principles of ROC analysis. Semin Nucl Med 8:283–298.

Morrison K, Papapetrou C, Hol FA, Mariman EC, Lynch SA, Burn J, Edwards YH. 1998. Susceptibility to spina bifida; an association study of five candidate genes. Ann Hum Genet 62:379–396.

Mudd SH, Finkelstein JD, Irreverre F, Laster L. 1964. Homocystinuria: an enzymatic defect. Science 143:1443–1445.

Mudd SH, Havlik R, Levy HL, McKusick VA, Feinleib M. 1981. A study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 33:883–893.

Mudd SH, Levy HL, Skovby F. 1995. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. New York: McGraw-Hill. p 1279–1327.

Mudd SH, Finkelstein JD, Refsum H, Ueland PM, Malinow MR, Lentz SR, Jacobson DW, Brattström L, Wilcken B, Wilcken DEL, Blom HJ, Stabler SP, Allen RH, Selhub J, Rosenberg IH. 2000. Homocysteine and its disulfide derivatives: a suggested consensus terminology. Arterioscler Thromb Vasc Biol 20:1704–1706.

Nelen WL, Blom HJ, Thomas CM, Steegers EA, Boers GH, Eskes TK. 1998. Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages. J Nutr 128:1336–1341.

Nygård O, Refsum H, Ueland PM, Vollset SE. 1998. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland homocysteine study. Am J Clin Nutr 67:263–270.

Ramsbottom D, Scott JM, Molloy A, Weir DG, Kirke PN, Mills JL, Gallagher PM, Whitehead AS. 1997. Are common mutations of cystathionine beta-synthase involved in the aetiology of neural tube defects? Clin Genet 51:39–42.

Refsum H, Ueland PM, Svardal AM. 1989. Fully automated fluorescence assay for determining total homocysteine in plasma. Clin Chem 35:1921–1927.

Refsum H, Ueland PM, Nygård O, Vollset SE. 1998. Homocysteine and cardiovascular disease. Annu Rev Med 49:31–62.

Rubba P, Faccenda F, Pauciullo P, Carbone L, Mancini M, Strisciuglio P, Carrozzo R, Sartorio R, Delgiudice E, Andria G. 1990. Early signs of vascular disease in homocystinuria—a noninvasive study by ultrasound methods in eight families with cystathionine-beta-synthase deficiency. Metabolism 39:1191–1195.

Sardharwalla IB, Fowler B, Robins AJ, Komrower GM. 1974. Detection of heterozygotes for homocystinuria. Study of sulphur-containing amino acids in plasma and urine after L-methionine loading. Arch Dis Child 49:553–559.

Silberberg J, Crooks R, Fryer J, Wlodarczyk J, Nair B, Guo XW, Xie LJ, Dudman N. 1997. Gender differences and other determinants of the rise in plasma homocysteine after L-methionine loading. Atherosclerosis 133:105–110.

Sperandeo MP, Candito M, Sebastio G, Rolland MO, Turccarel C, Giudicelli H, Dellamonica P, Andria G. 1996. Homocysteine response to methionine challenge in four obligate heterozygotes for homocystinuria and relationship with cystathionine beta-synthase mutations. J Inherited Metab Dis 19:351–356.

Stabler SP, Lindenbaum J, Savage DG, Allen RH. 1993. Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. Blood 81:3404–3413.

Steegers-Theunissen RP, Boers GH, Trijbels FJ, Finkelstein JD, Blom HJ, Thomas CM, Borm GF, Wouters MG, Eskes TK. 1994. Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? Metabolism 43:1475–1480.

Swift M, Morrell D. 1982. Cardiovascular risk in homocystinuria family members. Am J Hum Genet 34:1016–1018.

Tsai MY, Garg U, Key NS, Hanson NQ, Suh A, Schwichtenberg K. 1996. Molecular and biochemical approaches in the identification of heterozygotes for homocystinuria. Atherosclerosis 122:69–77.

Tsai MY, Welge BG, Hanson NQ, Bignell MK, Vessey J, Schwichtenberg K, Yang F, Bullemer FE, Rasmussen R, Graham KJ. 1999. Genetic causes of mild hyperhomocysteinemia in patients with premature occlusive coronary artery diseases. Atherosclerosis 143:163–170.

Ubbink JB, van der Merwe A, Delport R, Allen RH, Stabler SP, Riezler R, Vermaak WJ. 1996. The effect of a subnormal vitamin B-6 status on homocysteine metabolism. J Clin Invest 98:177–184.

Ulvik A, Refsum H, Kluijtmans LA, Ueland PM. 1997. C677T mutation of methylenetetrahydrofolate reductase gene determined in blood or plasma by multiple-injection capillary electrophoresis and laser-induced fluorescence detection. Clin Chem 43:267–272.

Wilcken DEL, Wilcken B. 1997. The natural history of vascular disease in homocystinuria and the effects of treatment. J Inherited Metab Dis 20:295–300.

Yap S, O'Donnell KA, O'Neill C, Mayne PD, Thornton P, Naughten E. 1999. Factor V Leiden (Arg506Gln), a confounding genetic risk factor but not mandatory for the occurrence of venous thromboembolism in homozygotes and obligate heterozygotes for cystathionine beta-synthase deficiency. Thromb Haemost 81:502–505.

Zweig MH, Campbell G. 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39:561–577.