Determinants and Vitamin Responsiveness of Intermediate Hyperhomocysteinemia ($\geq$ 40 μmol/liter)

The Hordaland Homocysteine Study

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Abstract

From 1992–93, we screened 18,043 subjects, aged 40–67 yr, and found 67 cases (0.4%) with total plasma homocysteine (tHcy) $\geq$ 40 μmol/liter. Compared to 329 controls, the cases had lower plasma folate and cobalamin levels, lower intake of vitamin supplements, consumed more coffee, and were more frequently smokers. Homozygosity for the C677T mutation in the methylenetetrahydrofolate reductase gene was observed in 73.1% of the cases and 10.2% of the controls. Only seven cases with cobalamin deficiency and one with homocystinuria received specific therapeutic instructions. 2 yr after the screening, 58 subjects were reinvestigated. 41 still had tHcy > 20 μmol/liter, and in 37 of these, intervention with low dose folic acid (0.2 mg/d) was started. Notably, 34 of 37 (92%) had homozygosity for the C677T mutation. Plasma tHcy was reduced in all but two after 7 wk, and became normal within 7 mo in 21 of 37 subjects. Most of the remaining subjects obtained a normal tHcy level with 5 mg/d of folic acid. We conclude that most subjects with hyperhomocysteinemia $\geq$ 40 μmol/liter in the general population have the C677T mutation combined with low folate status. Daily supplement of low dose folic acid will reduce and often normalize their tHcy level. (*J. Clin. Invest.* 1996. 98: 2174–2183.) Key words: homocysteine • methylenetetrahydrofolate reductase • genetics • folic acid • vitamin treatment

Introduction

Moderate elevation of homocysteine (Hcy)[1] in plasma is an independent risk factor for atherosclerotic disease in the coronary, cerebral as well as the peripheral arterial vessels (1, 2). This sulphur amino acid is formed from methionine during transmethylation, and is either salvaged to methionine by a folate and cobalamin-dependent remethylation reaction, or directed towards degradation by the vitamin B_6-dependent enzyme cystathionine β-synthase (CBS) (3). The total Hcy (tHcy) concentration in serum/plasma reflects cellular Hcy metabolism and is usually about 10 μmol/liter (4). Patients with inborn errors in CBS often have high plasma levels above 100 μmol/liter, i.e., severe hyperhomocysteinemia (5). Intermediate and moderate hyperhomocysteinemia refers to plasma tHcy levels in the range 31–100 μmol/liter and 15–30 μmol/liter, respectively (6). These are more common conditions, and are usually attributable to deficiencies of folate or cobalamin (7), but also occur in renal failure (6).

A C677T mutation in methylenetetrahydrofolate reductase (MTHFR) gene has been associated with reduced activity and increased thermolability of the enzyme (8). Homozygous individuals for this mutation often have elevated tHcy (9, 10). This is probably related to the obligatory role of the enzyme in the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the carbon donor in Hcy remethylation (3). The allele frequency of this mutation is high in the general population, and the prevalence of homozygosity is 5–10% in several studies on Caucasians. Notably, the C677T mutation has been associated with neural tube defect (11, 12) as well as coronary heart disease in some (13) but not all studies (Verhoef, P., F.J. Kok, L.F. Kluijtmans, H.J. Blom, H. Refsum, P.M. Ueland, and D.A.C.M. Kruyssen. 1996. Manuscript submitted for publication).

The association of hyperhomocysteinemia with disease motivates investigation of the cause and therapy of elevated tHcy. However, to date, there are only limited data available on the biochemical and genetic basis of intermediate hyperhomocysteinemia in the general population. In the Hordaland Homocysteine Study, plasma tHcy was determined in a population based sample of 18,043 subjects aged 40–67 yr (14). In this population, we detected 67 individuals with plasma tHcy $\geq$ 40 μmol/liter. In the present study, we report on the vitamin status and the prevalence of the C677T MTHFR mutation as well as the response to vitamin therapy in these highly selected cases.

Methods

Study population. From April 1992 to April 1993, University of Bergen in cooperation with the National Health Screening Service performed the Hordaland Homocysteine Study. This study included 12,594 subjects aged 40–42 yr, 683 aged 43–64 yr and 4,766 aged 65–67 yr, a total of 18,043 subjects. 67 individuals (0.37%) with tHcy levels $\geq$ 40 μmol/liter at the first screening (stage 1) were enrolled in the present study. Their age, sex, plasma tHcy, plasma methylmalonic

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1. *Abbreviations used in this paper:* CBS, cystathionine β-synthase; CE, capillary electrophoresis; Hcy, homocysteine; LIF, laser-induced fluorescence; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; OR, odds ration; PCR, polymerase chain reaction; RDA, recommended dietary allowance; tHcy, total homocysteine.

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Table I. Demographics and Median Blood Values of Controls and Categories of Study Population at Stage 1

Variable	Controls	All cases	Group I	Group II	Group III	Group IV	Group V
No. (*n*)	329	67	7	12	21	16	11
% male	48.6	62.7‡	42.9	50.0	66.7	68.7	72.7
Mean age, yr	53	49§	56	50	47	47	56
(range)	(40–67)	(40–67)	(40–67)	(40–67)	(40–67)	(40–66)	(40–67)
Smokers (%)	29.1	59.1§	71.4	75.0	55.0	56.2	45.5
Daily vit. suppl. (%)	13.3	2.0‡	0	0	6.2	0	0
No vit. suppl.	32.1	45.1*	50.0	90.0	50.0	30.8	66.7
Coffee consumption‖ (%)	33.4	44.8*	28.6	33.3	52.4	62.5	27.3
MTHFR status							
% TT	10.2	73.1§	0	66.7	95.2	81.2	72.7
%CT	38.1	19.4§	42.9	33.3	4.8	18.8	18.2
%CC	51.7	7.5§	57.1	0	0	0	9.1
Median tHcy	10.3	47.5§	49.5	46.8	44.6	50.8	46.1
(25–75 percentile)	(8.8–12.4)	(43.2–55.8)	(41.6–55.6)	(43.4–53.5)	(42.0–53.4)	(42.5–60.9)	(45.2–70.7)
Median folate	6.5	2.9§	7.2	2.0	2.6	3.0	3.1
(25–75 percentile)	(5.0–9.2)	(2.3–3.4)	(6.0–7.9)	(1.5–2.8)	(2.3–3.3)	(2.7–3.2)	(2.3–3.8)
Folate < 3.7 (%)	7.7	77.6§	0	100	85.7	87.5	72.2
Median Cbl	346	207§	64	189	241	205	207
(25–75 percentile)	(278–461)	(158–255)	(64–76)	(173–255)	(179–330)	(172–256)	(151–249)
Cbl < 150 (%)	1.8	19.4§	100.0	16.7	4.8	6.2	18.2
Median MMA		0.16	2.07	0.20	0.14	0.14	0.21
(25–75 percentile)		(0.12–0.26)	(0.68–2.75)	(0.11–0.26)	(0.13–0.20)	(0.10–0.16)	(0.13–0.26)
MMA > 0.27 (%)		22.7	100	16.7	19.0	0	20.0
Median Cr	91	92	86	88	102	90	95
(25–75 percentile)	(82–98)	(84–103)	(81–90)	(78–101)	(88–109)	(82–102)	(86–103)

Vit. suppl., vitamin supplement; tHcy, plasma tHcy (μmol/liter); Folate, plasma folate (nmol/liter); Cbl, plasma cobalamin (pmol/liter); MMA, plasma methylmalonic acid (μmol/liter); Cr, plasma creatinine (μmol/liter); *$P < 0.10$ vs. controls; ‡$P < 0.05$ vs. controls; §$P < 0.001$ vs. controls. ‖More than five cups of coffee/d.

acid (MMA), plasma creatinine, vitamin levels, and MTHFR status are listed in Table I.

Control subjects ($n = 329$) were recruited from the same study population (Table I), and comprised four to six males and females at each age increment of 1 yr from 40 to 67 yr of age.

The study was approved by the Regional Ethical Committee of Western Norway. Written informed consent was obtained from the cases, whereas the control population was anonymous to the investigators.

Protocol. Investigation, observation and vitamin treatment of the 67 subjects are divided into seven stages (Table II).

Stage 1 is the initial screening of the population of Hordaland Homocysteine Study.

Stage 2 represents the first follow up of the hyperhomocysteinemic subjects, taking place four to eight months after the initial screening. The subjects received a letter from the investigators, in which they were informed about the result of the tHcy determination, and they were asked to contact their own physician for a new medical examination. We were unable to reach four subjects due to death or change of address or name, leaving 63 persons. Blood for determination of plasma tHcy and vitamin status was obtained from 52 of 63 subjects (83%). At this stage, seven subjects were diagnosed as cobalamin deficient, based on plasma MMA > 0.27 μmol/liter and low plasma cobalamin, and the diagnosis was later confirmed by normalization of MMA and tHcy levels after administration of hydroxycobalamin. Of the remaining 56 subjects, one had homocystinuria due to CBS deficiency, and standard therapy with vitamin B_6, folic acid, cobalamin, and betaine was later initiated. In the other 55 subjects, no specific therapeutic instructions were given. Among these, 10 subjects were included in a clinical study (15) where they for a period of 1-2 mo received folic acid and/or cobalamin therapy. Three subjects started treatment for celiac disease diagnosed at this stage, and three subjects had ulcerous colitis or Crohn's disease. We did not interfere with therapy (usually cobalamin injections) started by the patient's own physician, and all patients were informed that a common cause of a high tHcy level was poor vitamin status.

Stage 3: about 2 yr after the primary screening, all available cases were invited to participate in a second follow-up to control their plasma tHcy level and vitamin status. The attendance rate was 92% (58 of 63). From 41 of 58 subjects, we also obtained information about intake of vitamin supplements or cobalamin injections. 41 subjects still had a tHcy > 20 μmol/liter. Among these, 37 were invited to

Table II. The Follow Up and Intervention in Subjects with Total Homocysteine (tHcy) ≥ 40 μmol/liter

Month/Year	Stage	Subjects still under investigation	tHcy < 20 μmol/liter	Therapy initiated at given stage			No further follow up*
				Cbl	FA 0.2 mg/d	FA 5 mg/d	
				No. of subjects			
Cbl therapy and observation phase							
4/92–4/93	1 (screening)	67	—				4
6/92–12/93	2 (first follow up)	63	5	7			3
12/94–1/95	3 (second follow up)	58	17				4
FA intervention phase							
1/95	3 (before FA)	37	—		37		
2/95	4 (week 7)	37	10	1‡		4	1
3/95	5 (week 15)	25	12			2	1
9/95	6 (week 30)	13	6			6	1
10/95	7 (week 37)	6	6				

We were able to reach 63 of 67 subjects with tHcy ≥ 40 μmol/liter. Only seven of these were considered by a specialist in hematology to require cobalamin therapy. The remaining subjects were followed for a period of 2 yr after the screening (observation phase). Subjects who still had tHcy > 20 μmol/liter at stage 3 received therapy with folic acid (FA), first with low dose (0.2 md/d), and if not responding, with high dose FA (5 mg/d) or Cbl injections. *These subjects still had elevated tHcy, but were not available for further investigations, had serious disease or dropped out of the study.‡In this subject, plasma cobalamin declined from 180 to 115 pmol/liter during the initial seven wk of low dose FA therapy. She received treatment with cobalamin injections, and tHcy was normalized.

participate in an intervention study with folic acid 0.2 mg/d, whereas the remaining four subjects were excluded due to malignant disease (two subjects), homocystinuria (one subject), or lack of compliance (one subject).

Stages 4–7: only the 37 subjects included in the intervention trial at stage 3 were examined at stages 4, 5, and 6 which refer to about 7, 15, and 30 wk after start of folic acid therapy, respectively. A subject participated in the study until tHcy decreased to < 20 μmol/liter (corresponding to 97.5 percentile of the control population), which is referred to as normalized in this study. In subjects where tHcy decreased less than 15% between consecutive stages, the folic acid dose was increased to 5 mg/d, and a new blood sample was collected after 7 wk. After 30 wk of low dose therapy, subjects who still had tHcy > 20 μmol/liter received high dose folic acid (5 mg/d) for 7 wk. At the end of this period (denoted Stage 7), a plasma sample was collected for the determination of tHcy and vitamin status.

Categorization of study subjects according to tHcy response. The cases were divided into five subgroups according to the change in tHcy through stages 1–7.

Group I is subjects with cobalamin deficiency. The diagnosis is based on clinical evaluation, a plasma cobalamin less than 150 pmol/liter, elevated plasma tHcy and MMA (> 0.27 μmol/liter) at stage 1, and normalization of both metabolites upon cobalamin supplementation.

Group II is subjects whose tHcy was normalized by stage 3. These subjects were also referred to as early responders.

Group III is subjects who still had elevated tHcy at stage 3. They were from stage 3 on given 0.2 mg folic acid daily, and tHcy decreased more that 15% between consecutive stages and was eventually normalized. These subjects are also referred to as sensitive responders.

Group IV is based on the same criteria as group III, except that tHcy did not or only marginally (< 15% between consecutive stages) respond to low dose folic acid of 0.2 mg/d. These subjects are also called poor responders.

The remaining individuals who did not fulfill the criteria of groups I–IV, are collectively referred to as group V.

Blood sample collection. Details have been given previously (14). Briefly, nonfasting blood samples were collected into tubes containing EDTA, chilled and centrifuged within 1–3 h. The plasma fraction and packed blood cells were separated and stored at −20°C until analysis.

Mutation analysis. DNA was extracted from packed blood cells. The blood stored at −20°C was thawed and 5 μl resuspended in 20 μl water. After 5 min, 200 μl of Dynabead DNA solution (Dynal AS, Oslo, Norway) was added, and the DNA extracted, as described by the supplier. The PCR reaction was performed according to the method of Frosst et al. (8), and generated a 198-bp fragment. The C677T mutation created a HinFI recognition sequence with a cleavage product of 175 bp.

The analysis of DNA fragments was performed with capillary electrophoresis (CE) in 30 cm fused silica capillary (75 μm i.d.) coated inside with linear polyacrylamide (16), and filled with 0.6% hydroxypropylmethylcellulose in 89 mM Tris-borate buffer, 2 mM EDTA, pH 8.3. SYBR Green I (dilution 1:30,000; Molecular Probes, Inc., Eugene, OR) was used for DNA staining (17). Capillary filling (20 psi for 50 s), DNA sample injection (20,000 V for 5 s), and the electrophoresis (200 V/cm) were carried out with a PRINCE autosampler (PRINCE Technologies, Emmen, Netherlands), coupled to an in-house built laser-induced fluorescence (LIF) detector. The detector was equipped with a sheath flow cell and an Argon laser (488 nm). The samples were injected in tandem at 2.5 min intervals, so the DNA fragments from three samples migrated simultaneously in the capillary. Capillary refilling with sieving matrix was conducted every 20 samples. This method was characterized by high sample throughput, and was verified by comparison with electrophoresis in 3% agarose gel (8). Details on the construction of the LIF detector and CE method will be given in a forthcoming article.

Biochemical analyses. Plasma tHcy was determined by a modification (18) of a fully automated method based on precolumn derivatization with monobromobimane followed by HPLC (19). The precision of the assay corresponds to a coefficient of variation of < 3%. MMA in plasma was analysed by CE-LIF following derivatization with 1-pyrenyldiazomethane (20). Cobalamin in plasma was determined with a microparticle enzyme intrinsic factor assay run on an IMx system (Abbott Laboratories, Abbott Park, IL), and folate in plasma and erythrocytes were assayed using the Quantaphase folate radioassay produced by Bio-Rad Laboratories (Hercules, CA).

Statistical analyses. Differences between cases and controls were

tested with the two-sample *t* test and the Pearson chi-square test. Nonparametric Kruskal Wallis combined with Mann-Whitney U tests were used for comparison among subgroups of cases. For analysis of difference in biochemical parameters at the different stages, we performed a Friedman nonparametric analysis of variance, and levels differing between stages were isolated by Wilcoxon signed rank test. The differences in folate status by MTHFR genotype among controls were evaluated by one way analysis of covariance. The differences in slopes of the relation between plasma tHcy and plasma folate by MTHFR status was tested by linear regression with an interaction term. Analyses were carried out with the BMDP statistical software package (Dixon, W.J., 1992. BMDP Statistical Software Manual. University of California Press, Berkeley, CA).

Multivariate analyses for proportions were carried out with case-control methods with the case defined as an individual belonging to the group with plasma tHcy ≥ 40 μmol/liter. Logistic regression analyses were stratified on age (40–45, 45–55, and 55–67 yr) and sex, and folate status when appropriate. Exact odds ratio estimates and *P*-values were used when asymptotic methods did not converge due to empty (missing) categories (LogXact-Turbo; CYTEL Software Corp., Cambridge, MA). Only two-sided tests were used, and *P*-values < 0.05 were considered significant.

Results

Plasma tHcy, vitamin, and MTHFR gene status in controls. The control subjects ($n = 329$) had a median plasma tHcy of 10.3 μmol/liter (6.1–20.9 μmol/liter, 2.5–97.5 percentile), median plasma folate of 6.5 (3.0–16.2) nmol/liter, and the median plasma cobalamin level was 346 (159–723) pmol/liter. The overall frequencies ($n = 323$) of homozygosity for the C677T mutation (TT genotype), the heterozygous CT genotype, and CC genotype without the mutation were 10.2%, 38.1%, and 51.7%, respectively (Table I). Thus, the mutated allele frequency was 29.3%. The frequency of TT genotype was significantly ($P = 0.007$) higher in the upper quartile of plasma tHcy (19.2%) compared to the lowest three quartiles (7.3%). After adjustment for age, sex, and smoking, mean plasma folate was significantly ($P < 0.01$) lower in the TT (5.6 nmol/liter) compared to the CT (7.4 nmol/liter), and CC (7.7 nmol/liter) genotypes. Moreover, plasma tHcy was negatively correlated with plasma folate (overall $R = -0.38$), and the relation was strengthened with the number of T alleles ($P < 0.005$; Fig. 1).

Characteristics and pretreatment blood parameters of study population. 67 out of 18,043 subjects had a tHcy ≥ 40 μmol/liter at first screening (stage 1), and were included. Their age, sex, genotype, vitamin status, and selected characteristics at stage 1 are summarized in Table I.

The hyperhomocysteinemic subjects had markedly lower plasma folate (median 2.9 nmol/liter), plasma cobalamin and lower intake of vitamin supplements, and consumed more coffee, and were more frequently smokers as compared to the control subjects (Table I).

The overall dominating genotype was TT (49 of 67, 73.1%); 13 of 67 (19.4%) were heterozygous CT, whereas only 5 subjects (7.5%) were of the CC genotype. This corresponds to an overall allele frequency of 82.8% for the C677T mutation. The relation between plasma folate and the plasma tHcy level for the cases and controls is shown in Fig. 1, which clearly demonstrates that the hyperhomocysteinemic subjects, especially those of the TT genotype, represent the left tail of the plasma folate distribution.

Table III shows that overall there was a strong and highly significant dose–response relationship between the number of

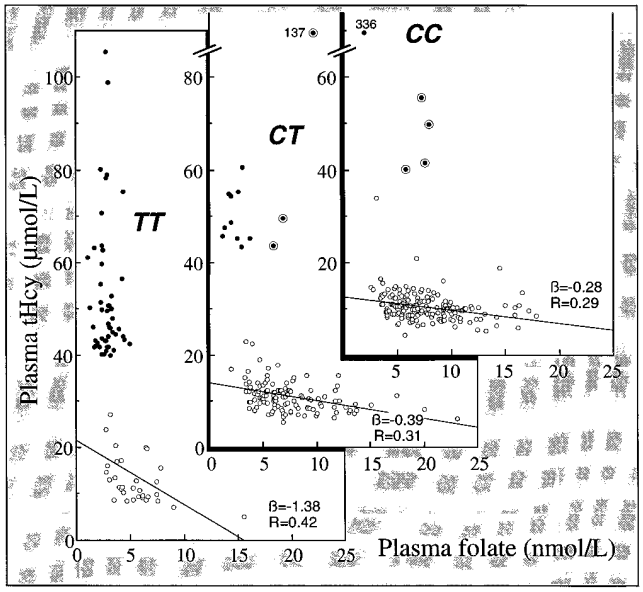

Figure 1. Plasma tHcy according to plasma folate level and MTHFR genotype in 67 cases with hyperhomocysteinemia and in 323 controls. Plasma tHcy is plotted against plasma folate at the initial screening (stage I) for cases (*filled symbols*) and controls (*open symbols*). Subjects (group I) later diagnosed as cobalamin deficient are marked as filled symbol surrounded by circle. The individual with tHcy of 336 μmol/liter (*upper left corner*, *left panel*) has homocystinuria. Separate plots are shown for homozygous TT subjects (*left panel*), heterozygous CT subjects (*middle*) and subjects without the mutation (CC subjects, *right*). The lines are obtained by linear regression of the data for the control subjects, and the slope increased ($P < 0.005$) with the number of T alleles.

T alleles and the likelihood of having tHcy above 40 μmol/liter (*P*-trend < 0.0001). The effect was strongest at folate levels less than 3.7 nmol/liter. At folate ≥ 3.7, there was little effect of the heterozygous CT state (odds ratio (OR) = 1.4, $P = 0.67$), but a highly significant OR of 10.1 for the homozygous TT state. Among individuals with plasma folate < 3.7, there was a strong effect not only of the TT state (OR = 175 compared to high folate and CC genotype), but also of the heterozygous CT state (OR = 55.1).

Significantly different OR at low and high plasma folate levels were found when the presence of one or two T alleles were compared to zero. At plasma folate < 3.7 nmol/liter, the OR for tHcy ≥ 40 μmol/liter was 36, and at plasma folate ≥ 3.7 nmol/liter, the OR was 3.1 (*P*-interaction = 0.049).

After exclusion of seven cases with cobalamin deficiency and one homocystinuria patient, the overall dose–response relationship was strengthened for the homozygous TT state at any folate level and of the heterozygous state at low folate levels (Table III).

Cobalamin-deficient subjects. Among the 63 subjects we were able to reach, only 7 were considered to have overt cobalamin deficiency (group I). At the screening, their median tHcy was 49.5 μmol/liter, plasma MMA was markedly elevated (median 2.07 μmol/liter) while plasma folate level was normal (median 7.2 nmol/liter). Their tHcy was essentially unchanged between the screening (stage 1) and the first follow-up (stage 2). From this time on, these subjects received regular cobal-

Table III. Odds Ratios for tHcy ≥ 40 μmol/liter by MTHFR Status and Plasma Folate Level

	All subjects				Subjects with plasma folate ≥ 3.7 nmol/liter				Subjects with plasma folate < 3.7 nmol/liter			
Genotype	Ctr (*n*)	Cases (*n*)	OR‡	*P*	Ctr (*n*)	Cases (*n*)	OR§	*P*	Ctr (*n*)	Cases (*n*)	OR§	*P*
CC	164	5	1	—	154	4	1	—	10	1	3.5	0.28
CT	121	13	2.9	0.07	115	4	1.4	0.67	6	9	55.1	< 0.0001
TT	33	49	15.0	< 0.0001	25	7	10.1	0.0005	8	42	175.4	< 0.0001
P-trend				< 0.0001				0.001				0.0001
CC*	164	0	1	—	154	0	1	—	10	0	—	—
CT*	121	10	14.1	0.004	115	1	1.4	0.84	6	9	254	< 0.0001
TT*	33	49	96.6	< 0.0001	25	7	51.6	< 0.0001	8	42	842	< 0.0001
P-trend				< 0.0001				0.001				0.0001

Ctr, controls; cases, subjects with plasma tHcy ≥ 40 μmol/liter; OR, odds ratios. *Seven cobalamin-deficient cases and one homocystinuria patient excluded. ‡Stratified on age, sex, and plasma folate. §Stratified on age and sex. Subjects with plasma folate ≥ 3.7 nmol/liter and of the CC genotype as reference.

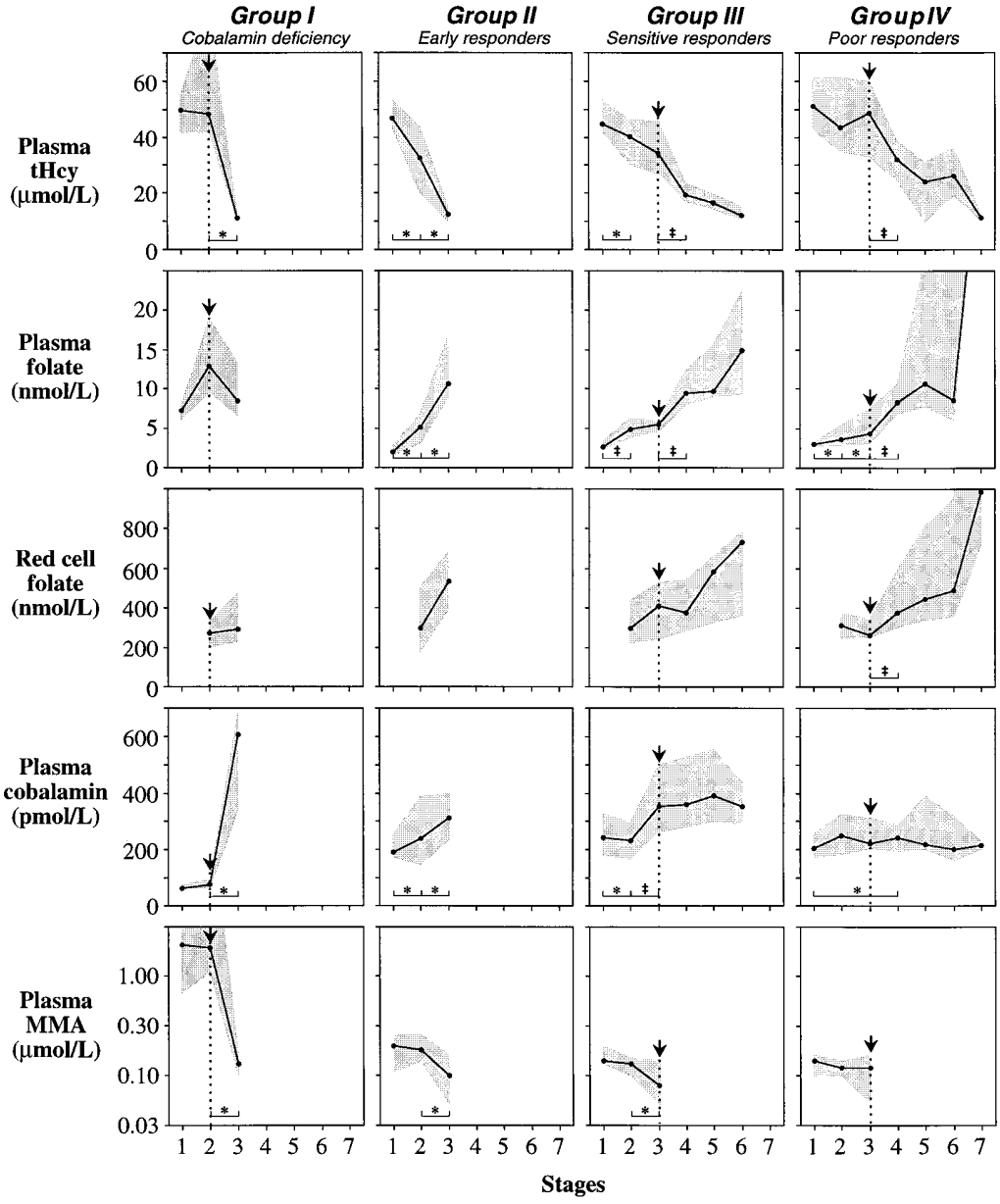

Figure 2. Changes in median tHcy and vitamin status during follow-up. Initiation of vitamin treatment (cobalamin injection in group I and 0.2 mg folic acid in groups III and IV) is indicated by arrows. Data are given as medians (*solid lines*) with interquartile ranges (*shaded area*). Group V subjects are not included. Complete data set was obtained for every subject for stages 1 to 3 in groups I and II, and for stages 1 to 4 in groups III and IV. For this time period, changes between stages were first analyzed by Friedman test. The consecutive intervals with significant changes were identified by the Wilcoxon's signed rank test. The significant levels are given over the vertical bars. *$P < 0.05$; ‡$P < 0.005$.

Table IV. Vitamin Use and Its Relation to Changes in Median Levels of Plasma tHcy, Vitamins, and Serum MMA from Stages 1 to 3

		Plasma tHcy			Plasma folate			Red cell folate		Plasma MMA			Plasma cobalamin			MTHFR status
		Stage														
Vitamin use	*n*	1	2	3	1	2	3	2	3	1	2	3	1	2	3	TT/CT/CC
		μmol/liter			*nmol/liter*			*nmol/liter*		*μmol/liter*			*pmol/liter*			*No.*
Cbl (Group I)	7	49.5	48.0	11.0*	7.2	12.9	8.4	271	292	2.07	1.93	0.13*	64	75	606*	0/3/4
Cbl	6	53.2	43.8	42.6	3.1	3.1	4.0	391	483	0.17	0.13	0.08*	183	204	741*	6/0/0
FA‡	11	50.4	42.9	22.9*	2.8	3.6	6.7*	315	429	0.18	0.12	0.09*	243	249	263	11/0/0
Cbl+FA	2	46.0	32.7	9.8	1.4	3.9	18.1	116	527	0.35	0.33	0.05	121	129	509	1/1/0
No vitamins	14	51.6	38.8	46.2	2.7	4.6	4.8*	318	261	0.14	1.12	0.09*	189	207	221*	10/4/0
No information	17	43.3	33.2	30.8*	2.9	6.3	5.8*	294	403	0.15	0.14	0.12	247	266	269*	15/2/0

From 41 out of 58 subjects, we obtained information about vitamin therapy between stages 2 and 3. The patient with homocystinuria is excluded. Cbl, cobalamin; MMA, methylmalonic acid; FA, folic acid. *Overall significant differences between the stages 1, 2, and 3 ($P < 0.05$, Friedman ANOVA). ‡FA refers to either use of folic acid tablets or multivitamin tablets. Both contain 0.1 mg folic acid per tablet.

amin injections, and both tHcy and plasma MMA became normal (Table IV; Fig. 2).

Plasma tHcy during the observation phase. The remaining 56 of 63 subjects received no specific therapeutic instructions, but their tHcy level and vitamin status were investigated at least once after the screening. During the 2 yr observation period from stage 1 to stage 3, plasma tHcy declined significantly (median tHcy, 47.2, 40.0, and 34.2 μmol/liter, $P < 0.001$), but there was marked individual difference unrelated to their tHcy level.

Notably, several subjects started to take vitamin supplements or their physicians began therapy with cobalamin injections after they had received information about their elevated tHcy level (Table IV). Subjects taking supplements containing folic acid (0.1 mg) had a substantial reduction in plasma tHcy level and improvement of their vitamin status. In contrast, cobalamin injections alone had marginal effect on the tHcy level, except in the seven subjects with overt cobalamin deficiency. Subjects who did not take vitamin supplements or receive cobalamin injections, showed an increase in their tHcy level between stages 2 and 3 (Table IV).

During the observation phase, only 12 out of the 56 subjects (21%) had obtained a tHcy level < 20 μmol/liter. These subjects (denoted early responders, group II) already had a marked decline in median tHcy from the screening (46.8 μmol/liter) to stage 2 (median 32.7 μmol/liter, $P < 0.05$). At stage 3, the median tHcy was 12.4 μmol/liter (different from stage 2, $P < 0.05$). All subjects had plasma folate < 3.7 nmol/liter at stage 1, and all showed a time-dependent increase in the folate level. Plasma cobalamin also increased, and a moderate decline was observed in plasma MMA (Fig. 2). In 8 of 12 subjects in group II, we obtained information indicating increased vitamin use or change in health since stage 1: two subjects had received treatment for celiac disease, both received cobalamin injections every third month, and they took folic acid (0.1–0.2 mg/d). Three other subjects had also started with folic acid containing supplements (0.1 mg/d), and two had participated in an experimental study in which they received folic acid and cobalamin for 4–8 wk (15). One person had reduced her alcohol consumption.

Intervention phase. Cases who at stage 3 still had elevated tHcy > 20 μmol/liter were recruited for an intervention study with folic acid. 41 subjects were contacted, and 37 subjects were healthy and willing to participate. At stages 1, 2, and 3 their median tHcy levels were 48.1, 42.4, and 35.4 μmol/liter, respectively ($P = 0.05$).

After 7 wk of therapy with low-dose 0.2 mg folic acid daily (stage 4), median plasma tHcy had declined to 22.4 μmol/liter ($P < 0.001$). To further evaluate this response, we stratified the subjects into two groups according to their tHcy level during the 2 yr observation phase (Fig. 3). One group consisted of 12 subjects with tHcy ≥ 40 μmol/liter at all stages 1, 2, and 3. Median tHcy did not change over this period. After seven wk of low-dose folic acid, all subjects obtained a lower tHcy level (mean reduction of 48%), and the median tHcy decreased from 58.2 to 31.2 μmol/liter ($P < 0.001$). In the other group ($n = 25$), we included subjects with tHcy < 40 μmol/liter at stage 2 and/or stage 3. Notably, also in this group, the median tHcy level was not significantly changed between stages 2 and 3 ($P = 0.52$). After seven wk with folic acid, 21 of 23 obtained a lower plasma tHcy level (mean reduction of 30%), and the median tHcy declined from 32.2 to 21.7 μmol/liter ($P < 0.001$). This analysis served to differentiate the tHcy reduction following low-dose folic acid from the regression towards the mean phenomenon (21).

The 37 subjects were also grouped according to the folic acid responsiveness (Fig. 2). Normalization of tHcy (< 20 μmol/liter) was obtained with 0.2 mg/d of folic acid in 21 subjects, and these are referred to as sensitive responders (group III). Plasma folate at stage 1 (median 2.6 nmol/liter) was below the normal limit of 3.7 nmol/liter in 18 subjects. Their plasma cobalamin was within normal range, but lower than in the controls. From stages 1 to 3, both median folate and cobalamin increased significantly ($P < 0.01$). In line with the improved vitamin status, their median tHcy level gradually declined to 34.2 μmol/liter at stage 3, and already after 7 wk of folic acid therapy, the median tHcy was 19.5 μmol/liter. Time to obtain normal tHcy varied markedly between individuals, and was 15–30 wk in about 50% of the cases.

Group IV is denoted poor responders ($n = 16$) because

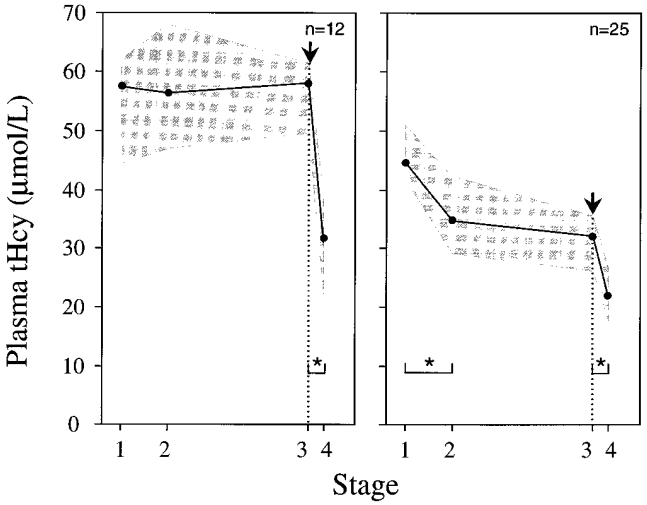

Figure 3. Changes in tHcy during the observation phase of two years (stages 1–3) and the initial 7 wk of the intervention phase (stages 3–4) with low dose folic acid. The left panel shows the effect of 0.2 mg/d folic acid in 12 subjects who at all three stages before intervention had tHcy > 40 μmol/liter. The right panel shows the effect of folic acid in 25 subjects in whom plasma tHcy < 40 μmol/liter at stages 2 and/or 3. Initiation of intervention with 0.2 mg folic acid is indicated by arrows. Data are given as medians (*solid lines*) and interquartile ranges (*shaded area*). The *x*-axes are drawn according to the time relation between the different stages. The changes in tHcy from stages 1–4 were first analyzed by the Friedman test, and significant changes were identified by the Wilcoxon's signed rank test. The significant levels are given over the vertical bars. $*P < 0.001$.

tHcy showed no or only marginal decrease (< 15%) between consecutive stages following low dose folic acid (Fig. 2). In contrast to the sensitive responders, the median tHcy of group IV subjects remained elevated and stable between stage 1 (50.8 μmol/liter) and stage 3 (median 48.5 μmol/liter), i.e. for a period of 2 yr. Some subjects (9 of 15) initially responded to 0.2 mg of folic acid/d with a > 15% reduction in tHcy level, but the tHcy level did not become < 20 μmol/liter. One subject had a marked decline in her plasma cobalamin (from 183 to 114 pmol/liter) between stages 3 and 4. Her tHcy level did not change and MMA was low (0.12 μmol/liter), but after start of cobalamin injections, she obtained a normal tHcy level. In 12 subjects, escalation of the folic acid dose to 5 mg/d efficiently reduced plasma tHcy to normal levels within 7 wk in all. The three remaining subjects dropped out before starting with high dose folic acid.

We sought differences in the vitamin status of sensitive and poor responders, which might explain the different folic acid requirement. At stage 3, median red cell folate was significantly ($P < 0.05$) lower in group IV than in group III (Fig. 2). The median cobalamin level also remained low (< 250 pmol/liter) in group IV subjects throughout all stages, but increased in group III (Fig. 2). Plasma cobalamin was significantly lower at stages 3 ($P = 0.02$) and 4 ($P = 0.005$) in group IV compared to group III.

The miscellaneous group. A total of 11 of 67 subjects did not adhere to the study protocol, and their tHcy was not normalized. This group (group V) includes four subjects who only participated at the screening, three subjects who also participated at stage 2 but then dropped out, and three subjects who were excluded from the intervention studies due to disease or noncompliance. It also includes a subject with homocystinuria who was healthy at the screening despite a tHcy level of 336 μmol/liter. His plasma methionine level was 575 μmol/liter. This subject rejected therapy for ~ 2 yr, but after suffering a mild cerebral stroke, he started with vitamin B_6 (450 mg/d), folic acid (10 mg/d), and betaine (12 g/d). His plasma tHcy is now between 40–50 μmol/liter.

Prevalence of TT genotype according to vitamin responsiveness. The mutated allele is unequally distributed among the different subgroups I–V. The cobalamin-deficient group I harbors only CT and CC genotypes. Among the early responders (group II), 8 of 12 (66.7%) were homozygous TT, and in the miscellaneous group V, 8 of 11 (72.7%) were homozygous (Table I). A remarkably high frequency of the homozygous TT genotype was found for the individuals with chronically elevated tHcy (groups III and IV). Among the sensitive responders (group III) and poor responders (group IV), 20 of 21 and 13 of 16 were homozygous, respectively, and there were no subjects without the mutation (CC genotype) (Table I). Thus, among the subjects treated with folic acid (groups III and group IV), the allele frequency for the C677T mutation was 94.6%.

Discussion

The 67 subjects under investigation represent 0.4% of 18,043 persons participating in the Hordaland Homocysteine Study (14), and were recruited on the basis of markedly elevated plasma tHcy (≥ 40 μmol/liter). The first objective of the present study was to record their baseline vitamin status, some lifestyle parameters and the C677T mutation in the MTHFR gene known to be associated with elevated plasma tHcy level (14). Then we followed the changes in tHcy level and vitamin status during an observation period of 2 yr where no specific therapeutic regimen was initiated, except for cobalamin injections in seven cobalamin-deficient subjects.

Notably, upon reexamination 2 yr later (stage 3), 41 of 58 (71%) subjects still had elevated tHcy (> 20 μmol/liter), and this fraction was 80% when excluding the seven patients treated for cobalamin deficiency. Our second objective was to investigate the response of the chronic hyperhomocysteinemia to folic acid close to RDA (0.2 mg/d) and eventually to a higher dose of 5 mg/d. The vitamin responsiveness was correlated to biochemical features and MTHFR status.

Characteristics of study population. Causes of elevated tHcy are genetic defects (22), impaired renal function (4), and lifestyle factors like smoking (14), heavy coffee consumption, (Nygård, O., H. Refsum, P.M. Ueland, I. Stensvold, J.E. Nordrehaug, G. Kvåle, S.E. Vollset. 1996. Coffee consumption and total plasma homocysteine. The Hordaland Homocysteine Study. In press.) low vitamin intake (23), and vitamin deficiencies. Among the vitamins, folate and cobalamin in serum/plasma show the strongest association with tHcy. The relation between vitamin B_6 and tHcy is weaker (23, 24), and optimal vitamin B_6 function seems of particular importance only to control tHcy following a methionine load (25–28).

The 67 hyperhomocysteinemic subjects had normal plasma creatinine levels which did not differ from the levels in controls (Table I), demonstrating that reduced renal function does not explain the elevated tHcy level. The cases, however, seem to

have a nutritional status and lifestyle pattern which were less favorable than in the control subjects, as judged by low vitamin intake, a high prevalence of smokers, and high intake of coffee (Table I). These life-style factors may contribute to, but certainly do not fully account for the intermediate hyperhomocysteinemia in the study population, because these commonly occurring factors are usually associated with only a moderate increase in tHcy (14).

A substantial fraction of the study population was vitamin deficient. The median plasma folate and cobalamin levels were 2.9 nmol/liter and 207 pmol/liter, which were significantly lower than in controls (Table I). Using the established lower reference limits for plasma folate (3.7 nmol/liter) and cobalamin (150 pmol/liter), 78% were folate deficient and 19% cobalamin deficient. The corresponding values for the controls were 7% and 1.8%. 23% (15 of 66) had elevated level ($>$ 0.27 μmol/liter) of the cobalamin marker MMA (7), but only in nine subjects (14%), high MMA was combined with low cobalamin level. Among these nine subjects, two had malabsorption. Thus, only the 7 subjects categorized as group I (10%) of 67 subjects with hyperhomocysteinemia were considered to have a deficiency state requiring regular cobalamin injections.

The most remarkable finding among the cases was the high prevalence of the homozygous genotype TT (73.1%) with an overall allele frequency of 82.8%. This is the highest frequency of the C677T mutation ever reported for any population. In the controls, the frequency of the homozygous genotype TT was 10.2%, and the mutated allele frequency was 29.3%, which equals the prevalence of the mutated gene reported previously for other unselected Caucasian populations (11, 13, 22, 29, 30).

Among the 49 hyperhomocysteinemic subjects of TT genotype, 88% were folate deficient (plasma folate $<$ 3.7 nmol/liter, Fig. 1). Also within the control population, the TT genotype was associated with significantly lower plasma folate than the levels found in CT and CC subjects. This suggests that homozygous subjects with low MTHFR activity are particularly susceptible to a negative folate homeostasis. Kluijtmans et al. (31) made a similar conclusion, based on their findings of a strong negative correlation between plasma folate and plasma tHcy which is confined to homozygous TT subjects. Likewise, Jacques et al. (29) demonstrated that individuals who are homozygous for the C677T mutation have elevated tHcy only when the plasma folate is in the lower normal range. In a population of 625 men, Harmon et al. (30) found 36% higher tHcy combined with lower plasma folate in individuals of the TT compared to the CC genotype, and an increasing frequency of TT genotype when the study population was ranked according to increasing tHcy levels. Our data from both controls and cases (Fig. 1) are in line with these findings. In addition, we demonstrate that at low plasma folate ($<$ 3.7 nmol/liter), even the heterozygous CT subjects have an increased likelihood of being hyperhomocysteinemic as compared to subjects without the mutation (Table III). Furthermore, the majority of the hyperhomocysteinemic subjects represents the extreme lower tail of a plasma folate distribution in a subsample of the TT genotype (Fig. 1). Conceivably, the folate dosing correcting the elevated tHcy in these subjects will probably suffice in most subjects with moderately elevated tHcy.

Folic acid responsiveness and requirements. High folic acid doses in the range 0.8 to 5 mg/d have been used in most intervention studies on Hcy reducing therapy, and the observation period has been between few days to several weeks (32–37). The low dose of 0.2 mg/d is close to the current RDA which has recently been demonstrated to be inadequate to maintain folate homeostasis and normalize tHcy in folate-depleted healthy subjects (38, 39). However, when added to the folate intake derived from food source, 0.2 mg/d may support folate homeostasis and therefore be relevant to dosing strategies aimed at disease prevention in large populations. Furthermore, such intervention in homozygous TT subjects with elevated tHcy also address the important question (29, 30) whether these individuals have an increased folate requirement.

In Norway, the amount of folic acid in vitamin supplements does not exceed 0.1 mg. Therefore, the reduced tHcy at stage 3 in cases taking folic acid supplements (Table IV), including seven cases classified as early responders (group II), suggests that low dose folic acid may normalize tHcy, at least in some individuals. All group II subjects were folate deficient at stage 1, and 8 of 12 were homozygous for the C677T mutation (Table I). However, assessment of effective folic acid dose from the tHcy reduction in early responders group II subjects is confounded by limited information about therapy and changes in lifestyle, but also by statistical regression of the extreme tHcy and folate values towards the mean (21).

We performed a folic acid intervention study on 37 subjects (groups III and IV) who had elevated tHcy at stage 3, and thus had permanent hyperhomocysteinemia lasting for at least 2 yr. The overall frequency of the C677T mutation in these subgroups was remarkably high, and 33 of 37 were homozygous. Their median tHcy level was 35.4 μmol/liter immediately before start, and after only 7 wk of therapy with 0.2 mg/d folic acid/d, the median tHcy declined to 22. 4 μmol/liter. Notably, this marked effect was related to reduction in tHcy in all but two subjects (mean reduction of $\sim$ 35%), and in 21 subjects (group III), tHcy reached the normal range of the control population ($<$ 20 μmol/liter) within 7 mo.

Considering that these hyperhomocysteinemic subjects represent the very extreme of the tHcy distribution, regression towards the mean should be taken into account when evaluating the changes in tHcy both in the observation phase and following intervention with low dose folic acid. Multiple measurements to obtain their true tHcy level may prevent misinterpretation (40–42), and we therefore evaluated the response relative to the tHcy in the observation phase, as shown in Fig. 3. All 12 patients with stable tHcy $>$ 40 μmol/liter during the 2 yr observation phase had substantial (mean 48%) decrease in tHcy following intervention. In the remaining 25 subjects receiving low-dose folic acid, there was an initial significant decline during the observation consistent with a regression phenomenon. Then tHcy was stable until the intervention which was associated with a significant decrease in tHcy. This subgroup analysis verifies the effect from 0.2 mg/d of folic acid on tHcy level.

Because marked tHcy reduction is obtained by adding low amount of folic acid to the daily intake, the tHcy response strongly suggests that hyperhomocysteinemia in most (21 of 37) TT subjects is related to low folate intake below or close to the RDA. Thus, most TT subjects probably efficiently utilize folate and do not require high doses.

In 16 subjects (group IV), we were unable to obtain a normal tHcy level with 0.2 mg of folic acid, but plasma tHcy rapidly responded to 5 mg folic acid/d. This is consistent with pre-

vious studies which have demonstrated that folic acid doses ≥ 1 mg/d effectively reduce tHcy in the majority of hyperhomocysteinemic subjects (24, 33, 35), including those with thermolabile methylenetetrahydrofolate reductase and intermediate hyperhomocysteinemia (10).

Unique genetic or biochemical features of the poor responders (group IV) may point to possible mechanisms behind the high folate requirement. A poor tHcy response to folate intake in a subgroup of individuals has recently been reported by Jacob et al. (38), and they suggested the possibilities of genetic differences or severe folate depletion in nonresponsive subjects. We found that both sensitive and poor responders (groups III and IV) had a very high but similar prevalence of homozygosity for the C677T mutation (90%).

Poor responders had higher tHcy and somewhat lower plasma folate and erythrocyte folate at stage 3 than sensitive responders, suggesting a more severe tissue folate depletion in these subjects. Moreover, poor responders had significantly lower plasma cobalamin level at the start of the intervention period (Fig. 2). Both sensitive and poor responders had normal plasma MMA, indicating normal cobalamin-dependent methylmalonyl-CoA activity in tissues. However, our data suggest a possible role of cobalamin metabolism during repletion of tissue folate stores, and may add support to the recommendation (22) on search for coexisting genetic variants involving cobalamin-related enzymes and receptors which may raise tHcy level.

Summary and conclusion. In 67 subjects with intermediate hyperhomocysteinemia, we found that about 10% had a typical cobalamin deficiency, whereas the hyperhomocysteinemia in the majority of the cases probably was related to the combined effect of low folate status and homozygosity for the C677T mutation. This study suggests that low dose of folic acid is associated with a substantial reduction or even normalization of elevated plasma tHcy in most subjects with intermediate hyperhomocysteinemia. These findings should influence strategies for the prevention of hyperhomocysteinemia in the general population. However, it remains to be seen whether such low dose regimen is effective in subjects with only marginally elevated tHcy level or in patients with various diseases. Furthermore, the longitudinal observational design does not control for change in lifestyle and intake of nutrients during the intervention period. The response to low dose folic acid should therefore be confirmed in controlled clinical trials.

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References

1. Boushey, C.J., S.A.A. Beresford, G.S. Omenn, and A.G. Motulsky. 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. *JAMA.* 274:1049–1057.

2. Perry, I.J., H. Refsum, R.W. Morris, S.B. Ebrahim, P.M. Ueland, and A.G. Shaper. 1995. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. *Lancet.* 346:1395–1398.

3. Finkelstein, J.D. 1990. Methionine metabolism in mammals. *J. Nutr. Biochem.* 1:228–237.

4. Ueland, P.M., H. Refsum, S.P. Stabler, M.R. Malinow, A. Andersson, and R.H. Allen. 1993. Total homocysteine in plasma or serum. Methods and clinical applications. *Clin. Chem.* 39:1764–1779.

5. Mudd, S.H., H.L. Levy, and F. Skovby. 1995. Disorder of transsulfuration. *In* The metabolic basis of inherited disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valles, editors. McGraw-Hill Inc., New York. 1279–1327.

6. Kang, S-S., P.W.K. Wong, and M.R. Malinow. 1992. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Annu. Rev. Nutr.* 12:279–298.

7. Allen, R.H., S.P. Stabler, D.G. Savage, and J. Lindenbaum. 1994. Metabolic abnormalities in cobalamin (vitamin-B12) and folate deficiency. *FASEB J.* 7:1344–1353.

8. Frosst, P., H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, G.H.J. Boers, M. den Heijer, L.A.J. Kluijtmans, L.P. van den Heuvel et al. 1995. Identification of a candidate genetic risk factor for vascular disease: A common mutation at the methylenetetrahydrofolate reductase locus. *Nat. Genet.* 10:111–113.

9. Engbersen, A.M.T., D.G. Franken, G.H.J. Boers, E.M.B. Stevens, F.J.M. Trijbels, and H.J. Blom. 1995. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocysteinemia. *Am. J. Hum. Genet.* 56:142–150.

10. Kang, S.-S., J. Zhou, P.W.K. Wong, J. Kowalisyn, and G. Strokosch. 1988. Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. *Am. J. Hum. Genet.* 43:414–421.

11. van der Put, N.M.J., R.P.M. Steegers-Theunissen, P. Frosst, F.J.M. Trijbels, T.K.A.B. Eskes, L.P. van den Heuvel, E.C.M. Mariman, M. den Heijer, R. Rozen, and H.J. Blom. 1995. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet.* 346:1070–1071.

12. Whitehead, A.S., P. Gallagher, J.L. Mills, P.N. Kirke, H. Burke, A.M. Molloy, D.G. Weir, D.C. Shields, and J.M. Scott. 1995. A genetic defect in 5,10 methylenetetrahydrofolate reductase in neural tube defects. *Q. J. Med.* 88:763–766.

13. Kang, S-S., E.L. Passen, N. Ruggie, P.W.K. Wong, and H. Sora. 1993. Thermolabile defect of methylenetetrahydrofolate reductase in coronary artery disease. *Circulation.* 88:1463–1469.

14. Nygård, O., S.E. Vollset, H. Refsum, I. Stensvold, A. Tverdal, J.E. Nordrehaug, P.M. Ueland, and G. Kvåle. 1995. Total plasma homocysteine and cardiovascular risk profile. The Hordaland homocysteine study. *JAMA.* 274:1526–1533.

15. Guttormsen, A.B., J. Schneede, P.M. Ueland, and H. Refsum. 1996. Kinetics of total plasma homocysteine in subjects with hyperhomocysteinemia due to folate or cobalamin deficiency. *Am. J. Clin. Nutr.* 63:194–202.

16. Hjertén, S. 1985. High-performance electrophoresis. Elimination of electroendosmosis and solute adsorption. *J. Chromatogr.* 347:191–198.

17. Skeidsvoll, J., and P.M. Ueland. 1995. Analysis of double-stranded DNA by capillary electrophoresis with laser-induced fluorescence detection using the monomeric dye SYBR green I. *Anal. Biochem.* 231:359–365.

18. Fiskerstrand, T., H. Refsum, G. Kvalheim, and P.M. Ueland. 1993. Homocysteine and other thiols in plasma and urine: Automated determination and sample stability. *Clin. Chem.* 39:263–271.

19. Refsum, H., P.M. Ueland, and A.M. Svardal. 1989. Fully automated fluorescence assay for determining total homocysteine in plasma. *Clin. Chem.* 35:1921–1927.

20. Schneede, J., and P.M. Ueland. 1995. Application of capillary electrophoresis with laser-induced fluorescence detection for routine determination of methylmalonic acid in human serum. *Anal. Chem.* 67:812–819.

21. Bland, J.M., and D.G. Altman. 1994. Regression towards the mean. *Br. Med. J.* 308:1499.

22. Motulsky, A.G. 1996. Nutritional ecogenetics: homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. *Am. J. Hum. Genet.* 58:17–20.

23. Selhub, J., P.F. Jacques, P.W.F. Wilson, D. Rush, and I.H. Rosenberg. 1993. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA.* 270:2693–2698.

24. Ubbink, J.B., W.J.H. Vermaak, A. van der Merwe, and P.J. Becker. 1993. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. *Am. J. Clin. Nutr.* 57:47–53.

25. Miller, J.W., M.R. Nadeau, D. Smith, and J. Selhub. 1994. Vitamin B-6 deficiency vs folate deficiency—comparison of responses to methionine loading in rats. *Am. J. Clin. Nutr.* 59:1033–1039.

26. Miller, J.W., J.D. Ribayamercado, R.M. Russell, D.C. Shepard, F.D. Morrow, E.F. Cochary, J.A. Sadowski, S.N. Gershoff, and J. Selhub. 1992. Effect of vitamin B-6 deficiency on fasting plasma homocysteine concentrations. *Am. J. Clin. Nutr.* 55:1154–1160.

27. Ubbink, J.B., W.J.H. Vermaak, A. van der Merwe, P.J. Becker, R. Delport, and H.C. Potgieter. 1994. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. *J. Nutr.* 124:1927–1933.

28. Franken, D.G., G.H.J. Boers, H.J. Blom, F.J.M. Trijbels, and P.W.C.

Kloppenborg. 1994. Treatment of mild hyperhomocysteinemia in vascular disease patients. *Arterioscler. Thromb.* 14:465–470.

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

30. Harmon, D.L., J.V. Woodside, J.W.G. Yarnell, D. McMaster, I.S. Young, E.E. McCrum, K.F. Gey, and A.S. Whitehead. 1996. The common "thermolabile" variant of methylenetetrahydrofolate reductase is a major determinant of mild hyperhomocysteinemia. *Q. J. Med.* 89:571–577.

31. Kluijtmans, L.A.J., L.P.W.J. VandenHeuvel, G.H.J. Boers, P. Frosst, B.A. VanOost, M. den Heijer, E.M.B. Stevens, J.M.F. Trijbels, R. Rozen, and H.J. Blom. 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.

32. Brattström, L.E., B.L. Hultberg, and J.E. Hardebo. 1985. Folic acid responsive postmenopausal homocysteinemia. *Metab. Clin. Exp.* 34:1073–1077.

33. Brattström, L.E., B. Israelsson, J.-O. Jeppsson, and B.L. Hultberg. 1988. Folic acid—an innocuous means to reduce plasma homocysteine. *Scand. J. Clin. Lab. Invest.* 48:215–221.

34. Brattström, L., B. Israelsson, B. Norrving, D. Bergqvist, J. Thörne, B. Hultberg, and A. Hamfelt. 1990. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease—effects of pyridoxine and folic acid treatment. *Atherosclerosis.* 81:51–60.

35. Naurath, H.J., E. Joosten, R. Riezler, S.P. Stabler, R.H. Allen, and J. Lindenbaum. 1995. Effects of vitamin B12, folate, and vitamin B6 supplements in elderly people with normal serum vitamin concentration. *Lancet.* 346:85–89.

36. Ubbink, J.B., P.J. Becker, W.J.H. Vermaak, and R. Delport. 1995. Results of B-vitamin supplementation study used in a prediction model to define a reference range for plasma homocysteine. *Clin. Chem.* 41:1033–1037.

37. Rasmussen, K., J. Møller, M. Lyngbak, A.-M. Holm Pedersen, and L. Dybkjaer. 1996. Age and gender specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. *Clin. Chem.* 42:630–636.

38. Jacob, R.A., M.M. Wu, S.M. Henning, and M.E. Swendseid. 1994. Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. *J. Nutr.* 124:1072–1080.

39. O'Keefe, C.A., L.B. Bailey, E.A. Thomas, S.A. Hofler, B.A. Davis, J.J. Cerda, and J.F. Gregory. 1995. Controlled dietary folate affects folate status in nonpregnant women. *J. Nutr.* 125:2717–2725.

40. Horns, E.K., P. Kanotsky, G. Shakarji, and E. Cotlove. 1970. Biological and analytical components of variation in long-term studies of serum constituents in normal subjects: II. Estimating biological components of variation. *Clin. Chem.* 16:1022–1027.

41. Williams, G.Z., D.S. Young, M.R. Stein, and E. Cotlove. 1970. Biological and analytical components of variation in long-term studies of serum constituents in normal subjects. I. Objective, subject selection, laboratory procedures, and estimation of analytic deviation. *Clin. Chem.* 16:1016–1021.

42. Gardner, M.J., and J.A. Heady. 1973. Some effects of within-person variability in epidemiological studies. *J. Chron. Dis.* 26:781–795.