Hyperhomocysteinemia in children with juvenile idiopathic arthritis is not influenced by methotrexate treatment and folic acid supplementation: A pilot study

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Abstract

Objective

Our first objective was to compare plasma total homocysteine (tHcy) concentrations in juvenile idiopathic arthritis (JIA) patients requiring methotrexate (MTX) treatment and healthy children. Our second aim was to evaluate the influence of low-dose (10-15 mg/m^2/week) MTX treatment combined with folic acid supplementation (1 mg/d) or placebo on tHcy concentrations in JIA patients.

Methods

In 17 JIA patients and 17 age- and sex-matched healthy children, baseline tHcy concentrations were measured. When MTX treatment was initiated, JIA patients were randomly assigned to folic acid 1 mg/d/p.o. followed by placebo (8 weeks each) or vice versa. Blood samples for measurement of tHcy, vitamin B_6, B_{12} and folate were taken after 4 weeks, 12 weeks and 20 weeks of treatment.

Results

1) In the healthy children the mean tHcy concentration was 6.3 ± 1.68 μmol/l as compared to 9.99 ± 5.17 μmol/l in JIA patients ($p < 0.04$). At baseline, 5/17 JIA patients had tHcy concentrations > 10.5 μmol/l, the 99th percentile for teenagers. 3/5 patients even exceeded the upper normal level for adults (tHcy ≥ 15 μmol/l). MTX treatment did not result in a significant increase of tHcy and folic acid supplementation had no significant impact on tHcy levels.

Conclusion

This pilot study shows that patients with JIA requiring MTX treatment have significantly elevated baseline plasma tHcy concentrations compared to age- and sex-matched healthy controls. No significant impact of MTX and folate supplementation on tHcy concentrations was found.

Key words

Homocysteine, folic acid, methotrexate, juvenile idiopathic arthritis, genetic polymorphisms.

Supported by the 1998 grant for "Research in Pediatric Rheumatology" donated by Prof. Josef Smolen, Vienna, Austria.

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Received on May 13, 2002; accepted in revised form on December 12, 2002.

Introduction

Homocysteine (Hcy) is a sulfur containing amino acid derived from the essential amino acid methionine. Hcy is either metabolized via vitamin B_6 dependent pathways to cysteine or transmethylated via folate and vitamin B_{12} dependent pathways to reform methionine (1) by the enzyme methionine synthase (MS, gene symbol: *MTR*) which requires methionine-synthase reductase (MSR, gene symbol: *MTRR*) to maintain functional activity (2, 3). The folate metabolite 5-methyltetrahydrofolate – derived by the enzyme 5,10-methylenetetrahydrofolate-reductase (MTHFR, gene symbol: *MTHFR*) – is an essential cofactor in the Hcy pathway (Fig. 1) (4). Hcy concentrations depend on age, sex and ethnic background (5). Hcy is a clinically relevant parameter: increased total Hcy (tHcy) plasma levels (referred to as tHcy levels > 15 µmol/l) (6) are associated with coronary artery disease, premature stroke and venous thrombosis (7-11). Furthermore there is increasing evidence indicating that even mild hyperhomocysteinemia (tHcy levels: 10 - 15 µmol/l) is related to vascular disease (6, 7).

Folate is one of the key factors in hyperhomocysteinemia. Insufficient vitamin supply as well as genetic polymorphisms in genes encoding for key enzymes of the Hcy pathway such as the TT genotype of the *MTHFR* 677C→T polymorphism, compound heterozygosity for the *MTHFR* 677T and *MTHFR* 1298C allele, the *MTR* 2756A→G polymorphism of the gene coding for MS, as well as *MTRR* 66A→G (12) and *MTRR* 997C→G (13) located in the gene encoding MSR are associated with mild and moderate hyperhomocysteinemia. The *MTHFR* 677C→T polymorphism and the compound heterozygous genotype for *MTHFR* 677T and *MTHFR* 1298C can result in an increased need for folate (4, 14). Other factors contributing to hyperhomocysteinaemia are immobility, smoking, chronic disease (e.g. hypothyroidism, renal failure or chron-

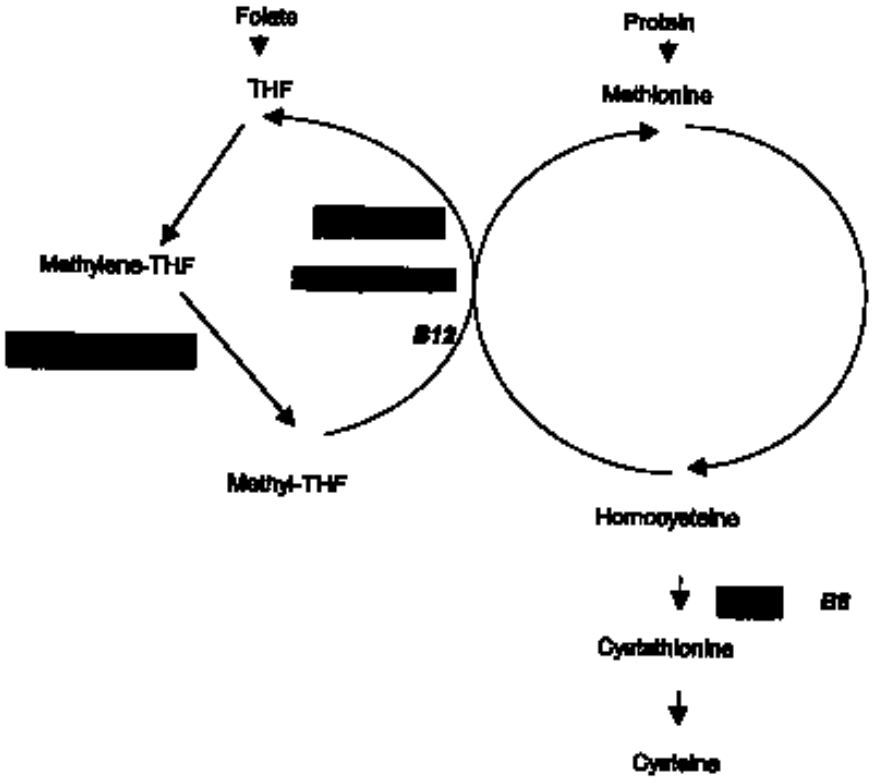

Fig. 1. The metabolic pathway of Hcy. THF = tetrahydrofolate.
Enzymes: MTHFR = methylene tetrahydrofolate reductase; MS = methionine synthase; MSR = methionine synthase reductase; CBS = cystathionine synthetase (gene symbols in brackets)

ic inflammatory diseases) (15).
Methotrexate (MTX), a widely used disease modifying drug in patients with juvenile idiopathic arthritis (JIA) (16) is a folic acid analogue and thus a potent competitive inhibitor of dihydrofolate reductase. It is known that in adults with rheumatoid arthritis (RA) or psoriasis, plasma folate pools become depleted during long-term low-dose treatment with MTX (17, 18) and hyperhomocysteinaemia has been observed (19-21). Interestingly, the incidence of cardiovascular disease in RA patients is higher than in healthy controls (22). Folic acid supplementation has been shown to alleviate hyperhomocysteinemia (18, 23, 24). In children Ravelli *et al*. showed in a retrospective survey a decline in adverse events in 43 JIA patients under MTX treatment when folinate was added (Hcy was not measured) (16). But the necessity of folate supplementation in children is still under discussion (25-27).
The aim of this study was to compare baseline plasma tHcy concentrations in children with severe JIA requiring MTX treatment to those measured in healthy children. The impact of MTX combined with placebo or folate on tHcy concentrations was investigated.

Materials and methods
The study protocol was approved by the local ethics committees of all participating institutions.

Subjects
Seventeen Caucasian patients seen in pediatric centers comprising three regions of Austria (10 from Vienna, 4 from Salzburg and 3 from Tyrol) participated in the study. All patients (2 males, 15 females, median age 10.7 years, range 4 to 16 years) fulfilled the revised classification criteria for juvenile idiopathic arthritis (28). Twelve patients had rheumatoid factor negative polyarthritis, one patient had extended oligoarthritis, 4 patients presented with persistent oligoarthritis. All patients were treated with nonsteroidal antirheumatic drugs (NSARDs) such as naproxen, piroxicam or indomethacin. Due to inadequate clinical response to NSARDs, all patients required second-line treatment and were eligible for MTX treatment. No other second-line drugs, systemic or intraarticular corticosteroids or any other drugs known to interfere with Hcy concentrations were used two months before and during the course of the study. Patients with other chronic diseases known to be associated with hyperhomocysteinaemia were not allowed to participate in the study. Two patients smoked between 3 and 7 cigarettes per day. Nine patients and their parents (6 from Vienna, 1 from Salzburg, 3 from Tyrol) gave their written informed consent for the analysis of polymorphisms in the Hcy pathway.
As controls, 17 age- and sex-matched children (2 males, 15 females, median age 10.4 years, range 4 to 16 years) without chronic disease or significant acute infection were recruited in one of the cooperating centers (Vienna). Additionally, tHcy concentrations in the JIA patients were compared to measurements from a sample of 3321 teenagers aged 13-14 published by Osganian *et al*. The 99th percentile Hcy concentration for 2498 Caucasian children is given with 10.5 µmol/l (mean 5.2 µmol/l ± 1.72) (5).

Study design
Baseline tHcy plasma levels were assessed in JIA patients and in age- and sex-matched controls (5). After baseline JIA patients were enrolled in a 20-week double blind-placebo controlled-crossover trial to evaluate the effect of folic acid supplementation (1 mg/d/p.o.) versus placebo (1 capsule/d/p.o.) during low dose MTX treatment. Patients were clinically examined monthly starting at the initiation of MTX (baseline). During the first 4 weeks of the trial, the weekly dose of MTX was increased from an average dose of 5 mg/m^2/week to the therapeutic dose of between 10 and 15 mg/m^2/week. At week 4 the patients were randomly assigned to the two arms of the study. They started either with 8 weeks daily ingestion of 1 mg of folic acid (week 4 to 12) followed by 8 weeks daily ingestion of placebo (week 12 to 20) or 8 weeks daily ingestion of placebo (week 4 to 12) followed by 8 weeks daily ingestion of 1 mg of folic acid (week 12 to 20). Blood samples were taken after 4 weeks of MTX dosage adjustment and subsequently every 8 weeks. The MTX dosage remained unchanged during the 16 weeks of the crossover trial and was taken as a single dose once a week.

Clinical parameters
At each visit the patients were clinically examined by one pediatric rheumatologist. The number of actively inflamed joints was assessed at each visit by summing all the joints with pain (tenderness, pain while moving), swelling or limited range of movement.

Laboratory parameters
Plasma and serum samples were taken after an overnight fast. Controls were tested once, JIA patients were tested at baseline, after 4 weeks and subsequently every 8 weeks until 20 weeks was reached, between 3 and 7 days after MTX ingestion. Blood samples for plasma Hcy assessment were separated immediately after collection and stored at -60°C until analysis. In cases where samples had to be transported, dry ice was used. Plasma tHcy was determined using a modified version of the gas chromatography-mass spectrometry (GS-MS) method described by Stabler *et al.* (29). In brief, 500 µl of Hcy standard was mixed with internal standard (3-phenylbutyrate) and reduced with 12.5% dithiothreitol. The solution was incubated for 30 min at 40°C and deproteinized by adding 100 µl of 72% trichloroacetic followed by centrifugation at 5,000 g for 10 min. Five hundred µl of the clear supernatant were mixed with 500 µl of n-propanolol-pyridin (4:1), 50 µl propylchloroformate and 1 ml of chloroform-propylchloroformate (100:1). The solution was centrifuged at 3,000 g for 10 min. The chloroform layer was evaporated to dryness and dissolved in 100 µl of chloroform-propylformate. One µl of the sample was subjected to GC-MS analysis.
Vitamin B_6 (pyridoxal-5'-phosphate) was measured using high performance liquid chromatography (HPLC) as described by Kimura *et al.* (30). Vita-

min B_{12} and folate were measured with Microparticle Enzyme Immunoassay (Abbott Imx® Analyzer, Abbott Laboratories, Abbott Park, Illinois, USA).
The complete blood cell count, ASAT, ALAT, gammaGT and creatinin were determined with an automated analyzer. The erythrocyte sedimentation rate (ESR) was measured according to standard laboratory procedures.

Restriction fragment length polymorphism analyses
Genomic DNA was isolated from citrated blood samples according to standard procedures. Identification of *MTHFR* 677C→T (31), *MTHFR* 1298A→C (32), and *MTR* 2756A→G (2) was performed as described. The presence of *MTRR* 997C→G (mutation at nucleotide position 628 according to GenBank accession AF 121207) and *MTRR* 66A→G (mutation at nucleotide position 4216 according to GenBank accession AF 121202) was investigated in a multiplex polymerase chain reaction (PCR) followed by restriction enzyme cleavage of PCR amplification products and electrophoresis through 6% polyacrylamide gels (Novex, San Diego, CA) followed by SYBR Green I Nucleid Acid gel Stain, Molecular Probes, Eugene, Oregon.

Statistical analysis
Variables are reported as means ± standard deviation (SD) for each measurement. Two-sided t-tests were used to test the mean of the baseline-values of tHcy in JIA patients, healthy controls and collectives reported in the literature (5). To evaluate the effect of folic acid supplementation on tHcy, ANOVA models were fitted taking into account the crossover design. Plasma tHcy concentrations at the end of each 8-week treatment period were analyzed using vitamin B_6, vitamin B_{12} and folate as covariates. The influence of folic acid supplementation and tHcy on ASAT and ALAT was investigated separately by ANOVA. Associations between the measured variables (tHcy, folate, vitamin B_6, vitamin B_{12}) were assessed by correlation analyses using Spearman's correlation coefficient. To investigate the time trend over the study period for the number of affected joints,leukocyte counts and ESR, linear regression analyses were conducted for each patient with the number of weeks since study entry as the independent variable. The SAS®-system (Release 8.01) was used for the statistical analyses (33).
Under the assumption of a mean standard deviation of differences of 5 with n = 17, the statistical power to prove a difference of 3 µmol/l of Hcy between measurements was 76%. A p-value < 0.05 was considered to indicate statistical significance.

Results

Baseline tHcy
The mean baseline plasma tHcy level of 9.99 ± 5.17 µmol/l in the 17 JIA patients was significantly different from the mean in 17 healthy controls (6.3 ± 1.68 µmol/l; $p < 0.04$, two-sided t-test) and significantly higher than the mean plasma tHcy measured in healthy white teenagers (5.2 ± 1.72 µmol/l; $p < 0.001$, one-sample t-test) but below the 99th percentile for this group (< 10.5 µmol/l) (5). Two out of 17 healthy controls (11.7%) and 5/17 (29.4%) JIA patients (mean age 12 years, mean joint count 15) had baseline tHcy concentrations exceeding the 99th percentile (> 10.5 µmol/l) for Caucasian teenagers (5). Three female patients (mean age 11.3 years) with polyarthritis (mean joint count 17.7) had tHcy values > 15 µmol/l (mean: 18.53 ± 5.04 µmol/l) at baseline. Excluding these patients, in the remaining 14 children (2 males, 12 females, mean age 10.3 years, mean joint count 17.2) the mean baseline tHcy (8.16 ± 2.92 µmol/l) was still significantly higher compared to the mean for white teenagers in the population described by Osganian *et al.* (5.2 ± 1.72 µmol/l; $p < 0.002$, one-sample t-test) (5).

Influence of MTX, folic acid, vitamin B_6 and B_{12} on tHcy
During MTX treatment, for the whole group of 17 patients the mean plasma tHcy levels did not increase significantly. Mean plasma tHcy concentrations were not influenced significantly by folic acid supplementation nor by vitamin B_6 or B_{12} levels. Vitamin B_6, B_{12} and folate values as measured during the study period were all within normal ranges (Table I). In the correlation analysis no significant association was detected considering tHcy, folate, vitamin B_6 and B_{12}. In the performed crossover analyses of variance, neither a significant sequence effect nor a significant period effect was found.
Outcome and safety of MTX treatment
Analyzing the time trend for the number of affected joints, leukocyte count and ESR over the study period, significant negative linear trends in 6, 3 and 4 patients respectively were found, indicating that the number of affected joints or the leukocyte count or ESR values decreased during the course of the study. For the whole group a decline in the number of affected joints and ESR was found, while the leukocyte count remained stable. In 3 patients, single elevated ASAT and ALAT values were measured on separate occasions. No correlation was

Table I. Means ± standard deviation of tHcy, folate, vitamin B6, and B12 concentrations.

	Baseline controls (n=17)	Baseline JIA patients (n=17)	After 4 weeks of dosage adjustment	After 8 weeks of placebo	After 8 weeks of folate	Normal ranges
tHcy µml/l	6.3 ± 1.68	9.99 ± 5.17	11.03 ± 5.68	10.08 ± 5.91	9.64 ± 5.94	≤ 10.5
Folate ng/ml	-	6.13 ± 1.37	6.24 ± 1.87	9.7 ± 3.42	14.9 ± 6.74	3.1-12.4
Vit B_6 nmol/l	-	49.56 ± 13.25	51.38 ± 12.46	42.44 ± 11.24	47.0 ± 10.48	> 30.5
Vit B_{12} pg/ml	-	581 ± 177	637 ± 222	770 ± 306	727 ± 301	223-1132

Table II. Genetic polymorphisms in the Hcy metabolic pathway and baseline tHcy concentrations in 8 patients with seronegative polyarthritis and one patient with extended oligoarthritis (n = 9).

Patient	Sex	Age	Joint count	MTHFR677C→T	MTHFR1298A→C	MTRR66A→G	MTR2756A→G	MTRR997C→G	tHcy µmol/l
1*	f	13	18	Het	Het	Het	WT	WT	24.3
2*	f	12	7	Het	Het	WT	WT	WT	16.3
3	m	14	34	Het	Het	Het	WT	WT	7.2
4	f	5	5	WT	Hom	Hom	WT	WT	10.0
5	f	11	20	Het	WT	Het	WT	WT	9.6
6	f	7	24	Het	WT	Het	WT	WT	8.9
7*	f	9	11	WT	Het	Het	Het	WT	15.0
8	f	14	-	WT	Het	Het	WT	WT	10.0
9	f	16	8#	WT	Hom	Het	Het	WT	8.8

Het: heterozygous; Hom: homozygous; WT: wild type.
*Baseline tHcy concentrations above the 99th centile for 13- to 14-year-old teenagers.
Patient with extended oligoarthritis.
(Combinations of) Polymorphisms associated with significantly elevated tHcy and cardiovascular disease risk.
Polymorphisms associated with slightly elevated tHcy and cardiovascular disease risk.

found between tHcy and liver enzymes (data not shown).

Genetic polymorphisms

Genetic polymorphisms were analyzed in 9 patients, including 3/5 patients with tHcy concentrations at baseline exceeding the 99th percentile. Five of the 9 patients (55.6%) were heterozygous for the *MTHFR* 677C→T mutation, a genotype associated with a moderate tHcy increase, and 3 patients were compound heterozygous for the *MTHFR* 677C→T and *MTHFR* 1298A→C polymorphisms, a genotype associated with significant elevation of tHcy levels. The *MTHFR* 1298A→C allele was identified in 7 of 9 patients (77.8%) *MTRR* 66A→G was found in 8 of 9 patients (88.9%); one patient had the homozygous genotype. Additionally, both the *MTHFR* 677C→T and *MTRR* 66A→G mutations were identified in 2 patients. *MTR* 2756A→G tested positive in 2 of 9 patients (22.2%), while no patient tested positive for *MTRR* 997C→G (Table II).

Discussion

In the present pilot study, plasma tHcy concentrations in patients with severe JIA requiring MTX treatment were significantly higher than in age- and sex-matched healthy controls. Additionally, tHcy concentrations were significantly higher in JIA patients compared to a sample of 2,498 Caucasian teenagers reported by Osganian *et al*. (5). The children included by Osganian *et al*. come from a different population than the patients in the present study, but the mean tHcy concentrations in the white subpopulation do not differ significantly from previously published smaller cohorts in European populations (34, 35). In 5/17 patients, plasma tHcy concentrations before MTX exceeded the 99th percentile as defined by Osganian *et al*. (5). There was no significant difference concerning age and joint count between these 5 patients and the 12 patients with tHcy below the 99th percentile. Three of these 5 patients gave their consent for polymorphism analysis. Two patients had the compound heterozygous genotype for MTHFR 677 C→T and MTHFR 1298 A→C, known to be associated with reduced enzyme activities and increased tHcy plasma concentrations (1). One patient presented with a genotype with a still unknown impact on tHcy concentrations (heterozygous for MTHFR 1298A→C, MTRR 66A→G and MTR 2756A→G respectively). On the other hand, 2 patients with normal tHcy concentrations too had genotypes known to be associated with moderately increased tHcy (heterozygosity for MTHFR 677 C→T and homozygosity for the MTRR 66A→G polymorphism respectively) (1, 36, 37). Therefore we conclude that genotype is only one component amongst others potentially elevating tHcy concentrations in this sample.

The impact of exogenous factors such as immobility, chronic inflammation, lifestyle and diet on tHcy have to be considered (15). There were no differences concerning ESR and the number of affected joints – both parameters reflecting inflammatory activity and degree of immobilization – between patients with elevated and low tHcy concentrations. Only two girls with low tHcy concentrations reported to be smokers,and alcohol intake was denied for the whole study population. Dietary factors were not assessed, but folate, vitamin B_6 and B_{12} concentrations were within normal ranges throughout the study period, thus indicating a sufficient nutritional vitamin supply.

THcy in plasma was measured before and during MTX treatment combined with placebo or folic acid supplementation. In adults, plasma tHcy levels above 10 µmol/l are associated with cardiovascular disease, venous thrombosis and premature stroke (7, 11, 19). In children associations between stroke, thrombosis and a history of premature cardiovascular disease and elevated tHcy concentrations have been shown (5, 38). In adults with RA, increased plasma tHcy levels before MTX treatment have been observed (39) and it is known that the incidence of cardiovascular disease in RA patients is higher than in healthy controls (15). Therefore the clinical relevance of monitoring tHcy concentrations seems evident.

In our study, tHcy concentrations in the JIA patients – which were significantly elevated at baseline – did not show an additional increase during MTX treatment. Folic acid supplementation did not influence tHcy concentrations. In the present study, the JIA patients were exposed to the targeted therapeutic MTX dosage without folic acid supplementation only for 8 weeks because of ethical considerations (in Austria folic acid supplementation in JIA patients on MTX treatment is recommended). In adults with RA and psoriatic arthritis under treatment with MTX without folate supplementation for only 4 weeks, an increase of tHcy and decrease of folate was observed (39). Van Ede *et al.* reported a significant increase of tHcy in adult RA patients after 48 weeks of MTX treatment without folate supplementation (40). In a 12-month trial in adults with RA, Morgan *et al*. showed that the folate stores declined during MTX treatment without folic acid supplementation after months (19). Therefore it seems to be unclear how long folate stores last and how long tHcy can be kept within normal ranges under MTX treatment without folate supplementation in different patient populations. Future studies should address the question of the time course of depletion of folate stores. During the trial the number of affected joints as well as the ESR declined, indicating the efficacy of MTX on the inflammatory process. We hypothesize that the physical activity of the patients might have increased by the positive effect of MTX treatment – a factor potentially compensating for an MTX-induced increase in tHcy concentrations.

In the present study, the widely recommended supplementation dosage of 1 mg/d of folic acid has been applied (25-27). However, the adequacy of this dosage, considering tHcy concentrations, is less than clear and it seems necessary to establish dosage-finding trials in the future.

In summary, we conclude that JIA patients requiring MTX treatment have elevated tHcy concentrations. Based on present knowledge of the impact of elevated tHcy concentrations, these patients possibly face a higher risk for the development of premature cardiovascular disease even before treatment with antifolates such as MTX. Hyperhomocysteinemia is probably induced via an interaction of exogenous factors such as immobility and the ongoing process of chronic inflammation with genetic factors (e.g. polymorphisms). Further studies are warranted to focus on the impact of MTX and folate supplementation on tHcy in JIA patients.

Acknowledgement

The excellent technical support of Josenato Ilas and of Corinna Eberle is gratefully acknowledged. The study medication (placebo and verum capsules) was provided by Solvay-Pharma, Klosterneuburg, Austria. We are indebted to Professor Josef Smolen for financial support of our work.

References

1. FÖDINGER M, BUCHMAYER H, SUNDER-PLASSMANN G: Molecular genetics of homocysteine metabolism. *Miner Electrolyte Metab* 1999; 25: 269-78.
2. LECLERC D, CAMPEAU E,GOYETTE P *et al*.: Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. *Hum Mol Genet* 1996; 5: 1867-74.
3. LECLERC D, WILSON A, DUMAS R *et al*.: Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. *Proc Natl Acad Sci USA* 1998; 95: 3059-64.
4. FÖDINGER M, HÖRL WH, SUNDER-PLASSMANN G: Molecular biology of 5,10-methyl-enetetrahydrofolate reductase. *J Nephrol* 2000; 13: 20-33.
5. OSGANIAN SK,STAMPFER MJ, SPIEGELMAN D *et al*.: Distribution of and factors associated with serum homocysteine levels in children. *JAMA* 1999; 281:1189-96.
6. AMERICAN SOCIETY OF HUMAN GENETICS / AMERICAN COLLEGE OF MEDICAL GENETICS TEST AND TRANSFER COMMITTEE WORKING GROUP: Measurement and use of total plasma homocysteine. *Am J Hum Genet* 1998; 63: 1541-3.
7. HANKEY GJ, EIKELBOOM JW: Homocysteine and vascular disease. *Lancet* 1999; 354:407-13.
8. REES MM, RODGERS GM: Homocysteinemia: Association of a metabolic disorder with vascular disease and thrombosis. *Thrombosis Research* 1993; 71: 337-59.
9. DEN HEIJER M, BLOM HJ, GERRITS WBJ *et al*.: Is hyperhomocysteinaemia a risk factor for recurrent venous thrombosis ? *Lancet* 1995; 345: 882-5.
10. MCCULLY KS: Homocysteine and vascular disease. *Nature Medicine* 1996; 2,4: 386-9.
11. CLARKE R, DALY L, ROBINSON K *et al*.: Hyperhomocysteinemia:An independent risk factor for vascular diseae. *N Engl J Med* 1991; 324: 49-1155.
12. VAN DER PUTNMJ, VAN DER MOLENEF, KLUIJTMANSLAJ *et al*.: Sequence analysis of the coding region of human methionine synthase: Relevance to hyperhomocysteinaemia in neural-tube defects and vascular disease. *QJM* 1997; 90: 511-7.
13. WILSON A, LECLERC D, ROSENBLATT DS, GRAVEL RA: Molecular basis for methionine synthase reductase deficiency in patients belonging to the cblE complementation group of disorders in folate/cobalamin metabolism. *Hum Mol Genet* 1999; 8: 2009-16.
14. VAN DER PUT NMJ, GABREELS F, STEVENS EMB *et al*.: A second common mutation in the methylenetetrahydrofolate reductase gene: An additional risk factor for neural-tube defects ? *Am J Hum Genet* 1998; 62: 1044-51.
15. SCHNEEDE J, REFSUM H,UELAND PM: Biological and environmental determinants of plasma homocysteine. *Semin Thromb Hemostas* 2000; 26: 263-79.
16. RAVELLI A, MIGLIAVACCA D, VIOLA S, RUPERTO N, PISTORIO A,MARTINI A: Efficacy of folinic acid in reducing methotrexate toxicity in juvenile idiopathic arthritis. *Clin Exp Rheumatol* 1999; 17: 625-7.
17. REFSUM H,HELLAND S, UELAND PM: Fasting plasma homocysteine as a sensitive parameter of antifolate effect:A study of psoriasis patients receiving low dose methotrexate treatment. *Clin Pharmacol Ther* 1989; 46: 510-20.
18. MORGAN SL, BAGGOTT JE, REFSUM H, UELAND PM: Homocysteine levels in patients with rheumatoid arthritis treated with low-dose methotrexate. *Clin Pharmacol Ther* 1991; 50: 547-56.
19. MORGAN SL, BAGGOT JE,LEE JY, ALARCON GS: Folic acid supplementation prevents deficient blood folate levels and hyperhomocysteinemia during long term, low dose methotrexate therapy for rheumatoid arthritis: Implications for cardiovascular disease prevention. *J Rheumatol* 1998; 25: 441-6.
20. VAN EDE AE, LAAN RFJM, BLOM HJ: Methotrexate in rheumatoid arthritis: An update with focus on mechanisms involved in toxicity. *Semin Arthritis Rheum* 1998; 27: 277-92.
21. LEEB BF, WITZMANN G, OGRIS E *et al*.: Folic acid and cyanocobalamin levels in serum and erythrocytes during low-dose methotrexate therapy of rheumatoid arthritis and psoriatic arthritis patients. *Clin Exp Rheumatol* 1995; 13: 459-63.
22. ROUBENOFF R, DELLARIPA P, NADEAU MR *et al.*: Abnormal homocysteine metabolism in rheumatoid arthritis. *Arthritis Rheum* 1997; 40: 718-22.
23. MORGAN SL, BAGGOTT, JE, VAUGHN WH *et al.*: The effect of folic acid supplementation on the toxicity of low-dose methotrexate in patients with rheumatoid arthritis. *Arthritis Rheum* 1990; 33: 9-18.
24. ORTIZ Z, SHEA B, SUAREZ-ALMAZOR ME, MOHER D, WELLS GA,TUGWELL P: The effi-

cacy of folic acid and folinic acid in reducing methotrexate gastrointestinal toxicity in rheumatoid arthritis. A meta-analysis of randomized controlled trials. *J Rheumatol* 1998; 25: 36-43.

25. STENGER AAME, HOUTMAN PM, BRUYN GAW: Does folate supplementation make sense in patients with rheumatoid arthritis treated with methotrexate ? *Ann Rheum Dis* 1992; 51: 1019-20.
26. DIJKMANS BAC: Folate supplementation and methotrexate. *Br J Rheumatol* 1995; 34: 1172-4.
27. ALARCON GS, MORGAN SL: Folinic acid to prevent side effects of methotrexate in juvenile rheumatoid arthritis. *J Rheumatol* 1996; 23: 2184-5.
28. PETTY RE, SOUTHWOOD TR, BAUM J *et al*.: Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban 1997. *J Rheumatol* 1998; 25: 1991-6.
29. STABLER SP, MARCELL PD, PODELL ER, ALLEN RH: Quantitation of total homocysteine, total cysteine and methionine in normal serum and urine using capillary gas chromatography-mass spectrometry. *Ann Biochem* 1987; 162: 185-96.
30. KIMURA M, KANEHIRA K, YOKOI K: Highly sensitive and simple liquid chromatographic determination in plasma of B6 vitaminers, especially pyridoxal 5'-phosphate. *J Chro - matogr A* 1996; 722: 296-301.
31. FROSST P, BLOM HJ, MILOS R *et al*.: A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase [letter]. *Nat Genet* 1995; 10: 111-13.
32. WEISBERG I, TRAN P, CHRISTENSEN B, SIBANI S, ROZEN R: A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab* 1998; 64:169-72.
33. SAS Institute Inc.: *SAS Procedures Guide*, Version 8.01, 2000, Cary NC, USA.
34. REFSUM H, WESTENBERG F, UELAND PM: Plasma homocysteine in children with acute lymphoblastic leukemia: Changes during a chemotherapeutic regimen including methotrexate. *Cancer Res* 1991; 51: 828-35.
35. GRAF WD, OLEINIK OE, JACK RM,EDER DN, SHURTLEFF DB: Plasma homocysteine and methionine concentrations in children with neural tube defects. *Eur J Pediatr Surg* 1996; 6: 7-9.
36. WILSON A,PLATT R,WU Q *et al*.: A common variant in methionine synthase reductase combined with low cobalamin (vitamin B_{12}) increases risk for spina bifida. *Mol Genet Metab* 1999; 67: 317-23.
37. BROWN CA, McKINNEY KQ, KAUFMAN JS, GRAVEL RA, ROZEN R: A common polymorphism in methionine synthase reductase increases risk of premature coronary artery disease. *J Cardiovasc Risk* 2000; 7: 197-200.
38. PRENGLER M, STURT N, KRYWAWYCH S, SURTEES R, LIESNER R, KIRKHAM F: Homozygous thermolabile variant of the methylenetetrahydrofolate reductase gene: A potential risk factor for hyperhomocysteinaemia,CVD and stroke in childhood. *Dev Med Child Neurol* 2001; 43: 220-5.
39. SLOT O: Changes in plasma homocysteine in arthritis patients starting treatment with low-dose methotrexate subsequently supplemented with folic acid. *Scand J Rheumatol* 2001; 30: 305-7.
40. VAN EDE AE, LAAN RF, BLOM HJ *et al*.: Homocysteine and folate status in methotrexate-treated patients with rheumatoid arthritis. *Rheumatology* 2002; 41: 658-65.