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Screening for Serum Total Homocysteine in Newborn Children

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Background: **Newborn screening for total homocysteine (tHcy) in blood may identify babies with vitamin B_{12} (B_{12}) deficiency or homocystinuria, but data on the causes of increased tHcy in screening samples are sparse.**

Methods: **Serum concentrations of tHcy, cystathionine, methionine, folate, and B_{12} and the methylenetetrahydrofolate reductase (*MTHFR*) 677C>T polymorphism were determined in 4992 capillary blood samples collected as part of the routine screening program in newborn children. Methylmalonic acid (MMA), gender (*SRY* genotyping), and the frequency of six cystathionine β-synthase (CBS) mutations were determined in 20–27% of the samples, including all samples with tHcy >15 μmol/L (n = 127), B_{12} <100 pmol/L (n = 159), or methionine >40 μmol/L (n = 154).**

Results: **The median (5th–95th percentile) tHcy concentration was 6.8 (4.2–12.8) μmol/L. B_{12} status, as determined by serum concentrations of B_{12}, tHcy, and MMA, was moderately better in boys than in girls. tHcy concentrations between 10 and 20 μmol/L were often associated with low B_{12}, whereas tHcy >20 μmol/L (n = 43) was nearly always explained by increased methionine. tHcy did not differ according to folate concentrations or *MTHFR* 677C>T genotypes. None of the babies had definite CBS deficiencies, but heterozygosity led to low cystathionine, increased methionine, but normal tHcy concentrations.**

Conclusion: **Increased tHcy is a common but not specific finding in newborns. The metabolite and vitamin profiles will point to the cause of hyperhomocysteinemia. Screening for tHcy and related factors should be further evaluated in regions with high prevalence of homocystinuria and in babies at high risk of B_{12} deficiency.**

The intention with newborn screening is to identify babies with serious and treatable conditions before symptoms arise *(1, 2)*. Newborn-screening programs differ among countries, but most programs screen for phenylketonuria and congenital hypothyroidism and represent an important investment for the prevention of death and disability *(1)*.

Newborn screening for homocystinuria attributable to cystathionine β-synthase (CBS)[6] deficiency is carried out in several countries and in some regions of the United States *(1, 3)*. CBS deficiency is an autosomal recessively inherited disorder of the transsulfuration pathway (Fig. 1). On the basis of data from metabolic newborn screening, the worldwide birth prevalence of CBS deficiency is ~1 in 300 000, but with marked regional differences, being more common in Ireland and New South Wales (~1:60 000) *(3)*. Recent data based on mutation analyses in newborn samples suggest that it may be far more common, i.e., 1:20 000 or even higher *(4–6)*. To date, more than 130 disease-associated mutations in the *CBS* gene have been identified *(7)*. CBS deficiency leads to markedly increased concentrations of homocystine in urine and total homocysteine (tHcy) and methionine in blood

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[6] Nonstandard abbreviations: CBS, cystathionine β-synthase; tHcy, total homocysteine; MTHFR, 5,10-methylenetetrahydrofolate reductase; B_{12}, vitamin B_{12} (cobalamin); MMA, methylmalonic acid; EIA, enzyme immunoassay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; g.mean, geometric mean; CI, confidence interval; OR, odds ratio; AUC, area under the curve; and CMR, cystathionine/methionine ratio (× 100).

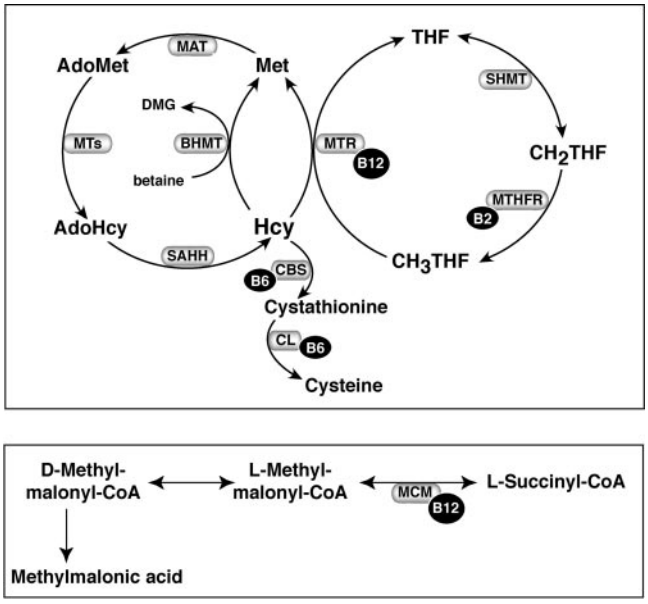

Fig. 1. Metabolic relationships.

(*Top panel*), homocysteine (*Hcy*) links the methionine (*Met*) cycle (*left*) with the folate cycle (*right*). Hcy is formed from methionine via numerous adenosylmethionine-dependent methyl transfer reactions (transmethylation). Hcy is either converted back to methionine (remethylation) or converted via cystathionine to cysteine (transsulfuration). The metabolism of Hcy depends on several B-vitamins including folate, B_{12}, B_6, and B_2. *MTs*, *S*-adenosylmethionine-dependent methyltransferases; *AdoMet*, *S*-adenosylmethionine; *MAT*, methionine adenosyltransferase; *SAHH*, *S*-adenosylhomocysteine hydrolase; *AdoHcy*, *S*-adenosylhomocysteine; *DMG*, dimethylglycine; *BHMT*, betaine-homocysteine *S*-methyltransferase; *CL*, cystathionine γ-lyase; *MTR*, methionine synthase; *THF*, tetrahydrofolate; *SHMT*, serine hydroxymethyltransferase; *CH_2THF*, 5,10-methylenetetrahydrofolate; *CH_3THF*, 5-methyltetrahydrofolate. (*Bottom panel*), relationship between MMA and B_{12}. Inborn errors or vitamin deficiency usually causes predictable changes in tHcy and related variables. *MCM*, methylmalonyl-CoA mutase.

(3, 8). In addition to a greatly increased risk of thromboembolic events, clinical signs and symptoms include mental retardation, psychiatric disorders, ectopia lentis, and skeletal abnormalities such as osteoporosis and marfanoid stature *(3)*. Approximately 50% of patients respond to pyridoxine with a marked decrease in tHcy. Independent of pyridoxine responsiveness, treatment from infancy with tHcy-lowering agents prevents premature vascular disease and mortality *(9, 10)*.

Homocystinuria may also be caused by severe deficiencies of methylenetetrahydrofolate reductase (MTHFR) or methionine synthase (Fig. 1), or defects in transport proteins or enzymes providing vitamin B_{12} (B_{12}) to methionine synthase. These so-called remethylation defects lead to increased tHcy, whereas methionine is low or within reference values *(11, 12)*. Symptoms often develop early in life and include developmental delay, failure to thrive, myelopathy, and sometimes, megaloblastic anemia *(11)*. The effect of therapy with B_{12}, folic acid, and betaine is variable *(11)*, but some data indicate that early diagnosis and treatment can reduce complications and delay symptom onset *(11)*.

Recently, an acquired cause of impaired remethylation has received increasing attention: many babies have low B_{12} concentrations *(13–15)*. Such neonatal B_{12} deficiency is nearly always attributable to low maternal B_{12} status *(16)*. These babies already have low B_{12} combined with increased concentrations of tHcy and the specific B_{12} marker, methylmalonic acid (MMA; Fig. 1) at birth. They continue to have a low B_{12} status during infancy, particularly if the baby is exclusively breastfed *(13, 17, 18)*. B_{12} deficiency may lead to failure to thrive and developmental delay in infancy and lower cognitive function later in childhood *(16)*. Low B_{12} status is usually easily corrected by supplying vitamin B_{12} *(19)*.

The conventional approach for newborn screening for homocystinuria is detection of increased methionine by the bacterial inhibition assay *(3)*. However, methionine measurement identifies only the more severe and usually pyridoxine-nonresponsive variants of CBS deficiency *(3)*; it does not detect inborn errors attributable to remethylation defects, nor will it identify babies with B_{12} deficiency. In this regard, measurement of tHcy may be a better approach. However, data on tHcy in newborn-screening samples are sparse *(20)*, and a critical evaluation of the use of tHcy measurements as a potential screening tool for identification of babies with homocystinuria or low B_{12} status is lacking.

In this study, we investigated tHcy and related variables in ~5000 newborn-screening samples. The effect of selected *CBS* mutations was also examined. Our intention was to identify the various factors determining increased tHcy concentrations in newborn babies and, if possible, to present some recommendations on the use of tHcy screening in newborns.

Materials and Methods

SAMPLES

From February to April 1999, approximately 12 000 capillary blood samples were sent to the Rikshospitalet University Hospital in Oslo for routine newborn screening for phenylketonuria and congenital hypothyroidism. From these samples, 4992 samples were randomly selected for the present study. The blood was collected in a gel separator tube, usually 3–5 days after birth. The tube was centrifuged locally, and the tube containing both serum and blood cells was then sent to the screening laboratory, where it was kept at 2–4 °C. Aliquots (~50–100 μL) of serum and packed blood cells from each sample were transferred into microtiter plates (52 plates; 96 samples per plate) after the routine screening had been completed, and the samples were then stored at −20 °C until analyses. For 104 babies (the Bergen sample set), we had information about time of blood collection relative to birth. All samples used in the study were unlinked and anonymous.

PROTOCOL

Total sample population. The total number of samples, i.e., microtiter wells, was 4992. However, one serum well was empty, and another six wells contained serum where the

blood tube had been sent from the local hospital without centrifugation. These seven samples were excluded from the analyses, leaving 4985 serum samples. Blood cells were available from all samples. Serum concentrations of tHcy, methionine, cystathionine, folate, and B_{12} and the *MTHFR* 677C>T genotypes were determined in all samples with sufficient volume available.

Random sample sets for MMA, gender, and CBS genotyping. These were random subsets of the total sample population for which we determined MMA, gender (n = 856; 17% of the total sample set), and *CBS* genotype (n = 1152; 23% of total sample set).

Samples with low B_{12} or increased tHcy or methionine. The random sets had <25 samples with B_{12} <100 pmol/L, tHcy >15 μmol/L, or methionine >40 μmol/L. To assess the biochemical relationships at these parts of the distributions, we measured MMA in all samples with B_{12} <100 pmol/L or tHcy >15 μmol/L, and *CBS* mutations were determined in all samples with tHcy >15 μmol/L or methionine >40 μmol/L. Gender determinations were carried out in samples with tHcy >15 μmol/L, B_{12} <100 pmol/L, or methionine >40 μmol/L.

Bergen sample set. In this sample set (n = 104), we knew the gender of the baby, the day after birth when the blood was collected, and the time from blood collection until the samples were frozen. This allowed us to assess differences in the serum variables according to the time since birth as well as the stability of the analytes during unfrozen storage.

BIOCHEMICAL METHODS

Measurement of MMA and amino acids. MMA was measured by a modified gas chromatography–mass spectrometry method based on ethylchloroformate derivatization *(21)*. tHcy was analyzed by two methods: an enzyme conversion immunoassay (EIA) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). tHcy measurement by EIA was carried out using a robotic sample processor *(22, 23)*. The CV for this assay is 6–8%, depending on concentration *(23)*. LC-MS/MS was used for analyzing tHcy, methionine, and cystathionine (Refsum et al., unpublished data). Briefly, after addition of reductant and deuterated standards {D,L-methionine-3,3,4,4-d_4; D,L-homocystine-3,3,3′,3′,4,4,4′,4′-d_8; and D,L-(2-amino-2-carboxyethyl)-homocysteine-3,3,4,4-d_4 [cystathionine-d_4]}, the sample was acid-precipitated, and the supernatant was injected on a C_{18} Supelco column [20 × 4 mm (i.d.); 5 μm bead size]. The column was equilibrated with 25 mmol/L acetic acid at a flow rate of 1 mL/min. The sulfur amino acids were eluted by an ethanol gradient (from 0% to 60% in 1 min, starting 0.5 min after the injection), and quantified by use of the transition from the precursor to the product ion for each of the amino acids (homocysteine, *m/z* 136→90; methionine, *m/z* 150→104; cystathionine, *m/z* 223→134) and their deuterated standards (homocysteine-d_4, *m/z* 140→94; methionine-d_4, *m/z* 154→108; cystathionine-d_4, *m/z* 227→138). The between-day CVs were 5–10%. The tHcy results obtained by EIA and LC-MS/MS gave nearly identical mean (SD) values: 7.5 (3.1) μmol/L and 7.6 (3.2) μmol/L, respectively. A Bland–Altman plot *(24)* revealed no differences in the two methods: $tHcy_{EIA} - tHcy_{LC\text{-}MS/MS} = 0.02(tHcy_{mean}) - 0.17$; $R = 0.03$). Thus, pooled data are presented.

Folate and B_{12} determination. Serum folate and B_{12} concentrations were measured by microbiological assays using a chloramphenicol-resistant strain of *Lactobacillus casei* and a colistin sulfate-resistant strain of *L. leichmannii*, respectively *(25, 26)*. Growth of *L. casei* responds to the biologically active folate species, including folic acid. The B_{12} assay measures total B_{12} in serum: the various cobalamin forms are released from transcobalamin and haptocorrins by boiling, and converted to cyanocobalamin, which is then used by the bacteria for growth. Both the folate and B_{12} assays were adapted to a microtiter plate format *(27)*, and carried out by a robotic workstation. The measurement range for B_{12} was 50–1000 pmol/L, and for folate it was 2–80 nmol/L. A result outside the range was set at the minimum or maximum measurable concentration.

Genotyping. Determination of male gender was based on identification of the *SRY* gene in the Y chromosome by real-time PCR *(28)*. The method was modified so it could be used on blood without DNA isolation *(29)* and was validated by *SRY* genotyping of the Bergen set, with known gender.

The *MTHFR* 677C>T polymorphism was determined by a real-time PCR technique using blood cells without previous isolation of DNA *(29)*.

We have previously identified six different mutations in the *CBS* gene among 10 Norwegian families with CBS deficiency: 785C>T, 797G>A, 833T>C, 919G>A, 959T>C, and 1105C>T *(30)*. These mutations were determined by a modification of a multiplex matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method *(31)* adapted for these genetic variants.

STATISTICAL ANALYSIS

Because the serum variables were skewed, the data were usually logarithmically transformed before further statistical analysis. If not otherwise stated, the variables are presented as geometric mean (g.mean) and 95% confidence intervals (CIs). The CIs were calculated on the logarithmic scale and then transformed back. For comparison between groups, the Student *t*-test for independent samples, ANOVA, analysis of covariance, or the χ^2 test was used. When significant differences among the means were observed, a post hoc test with Bonferroni correction was performed to identify significantly different group means. Simple correlations were performed with Spearman correlation coefficients. The dose–response relation-

ships between metabolites were also estimated with gaussian-generalized additive models *(32)*, as implemented in R *(33)*. This method generates a graphic representation of the relationship and allows adjustment for other covariates. Odds ratios (ORs) for increased tHcy concentrations were obtained by logistic regression analyses. The diagnostic usefulness of the various serum variables for the identification of low B_{12} status or CBS deficiency was assessed by use of ROC curves *(34)*. The areas under the ROC curves (AUC) were calculated (means and 95% CIs) to compare the diagnostic performance of the different variables. A two-tailed P value <0.05 was considered statistically significant. Data were analyzed using SPSS 11.0 (SPSS Inc.).

Results

The methods and analyses used are listed in Table 1. The serum volume available was only 50–100 μL, but results were obtained in >94% of the samples selected for analyses except for MMA, for which there was insufficient volume in ~20% of the samples.

SERUM VARIABLES AND REFERENCE INTERVALS

The mean, g.mean, and distributions of the serum variables are listed in Table 2. Comparisons of the mean with the g.mean and median (50th percentile) showed that the variables are skewed toward higher concentrations.

Reference limits are usually based on the 2.5th–97.5th percentile interval in an assumed healthy population. Our study population also included babies who were ill, premature, and/or who received nutrient intervention or medical treatment. We therefore used the 5th and 95th percentiles as reference limits. With such thresholds, the upper limits for tHcy and MMA were ~13 μmol/L and 0.60 μmol/L, respectively, whereas the lower limit for B_{12} was ~125 pmol/L. Almost the same thresholds were found when we used the 2.5th–97.5th percentile interval in a reference population confined to those with normal concentrations of factors (folate, B_{12}, and/or metabolites) known to influence the variable (data not shown).

GENDER EFFECTS

Vitamin B_{12} status was lower in girls than in boys, as suggested by lower B_{12} (g.mean = 299 vs 345 pmol/L; P <0.001), higher tHcy (7.1 vs 6.7 μmol/L; P = 0.036), and higher MMA concentrations (0.27 vs 0.25 μmol/L; P = 0.016). In line with this, the reference limits differed between the genders for some markers, including the lower limit for B_{12} (123 pmol/L for girls and 152 pmol/L for boys) and the upper limits for MMA (0.70 μmol/L for girls and 0.48 μmol/L for boys) and tHcy (13.9 μmol/L for girls and 12.1 μmol/L for boys). For cystathionine, the upper limit did not differ markedly between girls and boys, but the lower limit did, i.e., being 0.22 μmol/L for boys and 0.27 μmol/L for girls.

SIMPLE CORRELATIONS AND DOSE RELATIONSHIPS BETWEEN THE SERUM VARIABLES

Spearman correlations between tHcy and five other serum variables are listed in Table 3. Methionine, tHcy, and cystathionine were strongly correlated with each other. B_{12} was strongly inversely associated with tHcy, cystathionine, and MMA, whereas folate showed a relatively strong inverse correlation with methionine and cystathionine but was only weakly associated with tHcy.

The associations between these variables were further investigated by use of gaussian-generalized additive regression, which produces dose–response curves adjusted for other variables. For many associations, nonlinear relationships became apparent (Fig. 2). For example, methionine and tHcy were strongly associated with cystathionine, but not above their upper reference limits, and the predominant effect of B_{12} in relation to tHcy, MMA, and cystathionine was seen at low B_{12} concentrations. Several models other than those shown in Fig. 2 were investigated, but adjustment for different variables had usually no effect on the strength or pattern of association. Folate was associated to tHcy after exclusion of methionine from the model; a weak inverse correlation then became apparent (data not shown). As shown in Table 3, we found a weak inverse correlation between B_{12} and methionine, whereas the data presented in Fig. 2 indicated a weak positive correlation. This change in direction became apparent after tHcy was included in the model.

SAMPLES WITH INCREASED tHcy

The vitamin and metabolite concentrations in samples with increased tHcy compared with those with tHcy concentrations within the referenced interval are shown in Table 4. As tHcy increased, marked changes in g.mean concentrations and in proportion with abnormal concentration occurred for all serum variables except for folate. The changes in B_{12}, cystathionine, and MMA were most pronounced between normal to moderately increased concentrations of tHcy. In contrast, methionine increased throughout the observation range (Table 4 and Fig. 2). As tHcy increased above 15 μmol/L, increased methionine became the most common finding, and in samples with tHcy >20 μmol/L, low B_{12} was usually found only in combination with increased methionine.

In logistic regression analyses, low B_{12} (below the 5th percentile) was associated with tHcy >10 μmol/L [OR = 3.08 (95% CI, 2.28–4.18)], but less and nonsignificantly with tHcy >20 μmol/L [2.10 (0.62–7.14)]. The corresponding ORs for high methionine (above the 95th percentile) were 5.28 (4.00–6.96) and 20.73 (10.62–40.47), respectively.

DETECTION OF LOW B_{12} STATUS

Using ROC analysis, we compared tHcy with other variables related to B_{12} status (B_{12}, MMA, and cystathionine) in their ability to identify babies with low B_{12} status. For tHcy, cystathionine, and MMA, it was assumed that a

Table 1. Methods and analyses.

Variable measured	Method (Refs.)	Volume used, μL	Samples selected for analysis[a]	Analyses performed (% of selected)	Comment
Folate	MBA[b] *(25, 27)*	4	All samples	4947 (99.2)	
Vitamin B_{12}	MBA *(26, 27)*	19	All samples	4874 (97.8)	B_{12} <100 pmol/L: n = 159[c]
Methionine				4725 (94.8)	Met >40 μmol/L: n = 154[c]
Cystathionine	LC-MS/MS[d]	15	All samples	4726 (94.8)	
tHcy				4712 (94.5)	n_{pooled} = 4985 (100.0%)[e] $tHcy_{pooled}$ >15 μmol/L: n = 127[c]
tHcy	EIA *(22)*	5	All samples	4831 (96.9)	
MMA	GC/MS *(21)*	15	Random sample	708 (82.8)	n = 993 (19.9%)[e]
			tHcy >15 μmol/L	97 (76.4)	
			B_{12} <100 pmol/L	129 (81.1)	
			Bergen set	92 (88.5)	
MTHFR 677C>T	Real-time PCR *(29)*	1	All samples	4962 (99.5)	
Gender (*SRY*)	Real-time PCR *(28)*	1	Random sample	847 (99.1)	n = 1291 (25.9%)[e]
			tHcy >15 μmol/L	124 (97.6)	
			B_{12} <100 pmol/L	158 (99.4)	
			Met >40 μmol/L	154 (100.0)	
			Bergen set	101 (96.2)	
CBS mutations	MALDI-TOF MS *(31)*	5	Random sample	1133 (98.4)	n = 1327 (26.6%)[e]
			tHcy >15 μmol/L	123 (96.9)	
			Met >40 μmol/L	149 (96.8)	

[a] There were a total of 4985 serum samples and 4992 samples with blood cells (DNA analyses). The random samples for determination of MMA and gender included 856 samples, and determination of *CBS* mutations included 1152 samples. The Bergen set included 104 samples.

[b] MBA, microbiological assay; GC/MS, gas chromatography–mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

[c] Used as selection criterion for determination of MMA, gender, and *CBS* mutations (see *Materials and Methods*).

[d] See *Materials and Methods* for description of the method for methionine, cystathionine, and tHcy.

[e] Total number of analyses for a given analyte (proportion of total population).

Table 2. Descriptive statistics for the serum variables.

Variable	n	Mean (SD)	g.mean (95% CI)	Percentiles								
				2.5	5	10	25	50	75	90	95	97.5
Vitamin B_{12}, pmol/L	4874	355 (203)	305 (300–310)	81	123	158	218	308	434	625	815	1000
Folate, nmol/L	4947	20.3 (12.0)	17.6 (17.3–17.9)	6.5	7.5	9.3	12.7	17.4	24.1	34.3	43.0	55.3
Methionine, μmol/L	4725	22.2 (8.6)	20.5 (20.2–20.7)	8.7	10.5	12.5	16.2	21.3	26.7	32.5	37.3	41.8
tHcy, μmol/L	4985	7.5 (3.0)	7.0 (7.0–7.1)	3.8	4.2	4.7	5.6	6.8	8.6	10.9	12.8	15.1
Cystathionine, μmol/L	4726	0.60 (0.44)	0.52 (0.51–0.53)	0.20	0.23	0.28	0.37	0.50	0.71	0.97	1.24	1.53
MMA,[a] μmol/L	708	0.31 (0.31)	0.26 (0.25–0.27)	0.13	0.15	0.17	0.20	0.24	0.32	0.44	0.59	0.91

[a] In the random sample set.

baby had low B_{12} status when the two other metabolites were in the upper quartile and B_{12} was in the lower quartile. For B_{12}, a low B_{12} status was defined as tHcy, cystathionine, and MMA being in the upper quartile. "Normal B_{12} status" was defined as tHcy, cystathionine, and MMA being in the three other quartiles. When we used this definition to separate the normal from the abnormal, tHcy (AUC = 0.86) and cystathionine (AUC = 0.85) discriminated nonsignificantly better than MMA (AUC = 0.82), which in turn was marginally better than B_{12} (AUC = 0.78). Using the best sensitivity–specificity pairs, we obtained thresholds of ~8 μmol/L for tHcy, ~0.30 μmol/L for MMA, ~0.55 μmol/L for cystathionine, and ~220 pmol/L for B_{12}.

The relationships between serum B_{12} and the proportions with increased MMA, cystathionine or tHcy are shown in Fig. 3. The proportion with low B_{12} according to tHcy is also shown.

MTHFR 677C>T polymorphism, folate, and tHcy

The proportions with the CC, CT, or TT genotypes were 51.4%, 40.3%, and 8.4%, respectively, and the prevalence of the TT genotype was similar in boys and girls (8.9% vs 7.7%; $P = 0.54$). The g.mean tHcy in CC, CT, and TT genotypes was 7.0, 6.9, and 7.1 μmol/L, respectively ($P > 0.05$). The corresponding values for serum folate were 17.8, 17.4, and 17.8 nmol/L ($P > 0.05$). At folate <5 nmol/L, g.mean tHcy was moderately increased compared with babies with folate >5 nmol/L (g.mean = 7.7 vs 7.0 μmol/L; $P = 0.044$), but even at such folate concentrations, *MTHFR* genotypes had no effect on the tHcy concentrations.

Findings in samples with very low or high concentrations of vitamin or metabolites

Screening often focuses on samples with extremely high or low concentrations. As shown in Fig. 2, at either end of the distribution, the dose–response relationships may change. The findings for the samples with extremely low or high values of each of the analytes are summarized in Table 5. For the metabolites, the extreme group was confined to 10–13 samples, corresponding to the top or bottom 0.2% for the sulfur amino acids and 1.4% for MMA. For the vitamins with restricted measurement range, the extreme groups included a larger number of samples. For some extreme groups, the associations were unexpected. Thus, in samples with extremely low methionine, tHcy was within the reference interval, cystathionine was significantly increased, and folate was markedly increased. A similar pattern was seen in the samples with the highest folate concentrations, i.e., low methionine, but concentrations within the reference intervals for the two other amino acids. In samples with extremely increased cystathionine, the other variables were within the reference intervals, suggesting a metabolic defect distal to cystathionine. A surprising finding was observed in the samples with the lowest B_{12} concentrations: MMA

Table 3. Spearman correlations between serum variables.[a]

	Folate	Methionine	tHcy	Cystathionine	MMA[b]
Vitamin B_{12}	0.11	−0.03[c]	−0.32	−0.23	−0.28
Folate		−0.19	−0.06	−0.16	0.00[d]
Methionine			0.28	0.33	0.18
tHcy				0.36	0.33
Cystathionine					0.27

[a] If not otherwise stated, the correlation was significant at a level of $P < 0.001$.
[b] Correlations including MMA are based on the random sample set, n = 708.
[c] $P = 0.030$.
[d] $P = 0.97$.

was within the reference interval, methionine was nonsignificantly lower, tHcy was significantly lower, and cystathionine was significantly higher than in the remaining group.

CBS mutations and polymorphisms

The frequency of samples with a *CBS* mutation in this cohort is 2.47% *(6)*. We found that a *CBS* mutation was associated with lower cystathionine and higher methionine concentrations, whereas tHcy did not differ (Table 6). Accordingly, the ratio between cystathionine and methionine × 100 (CMR) was significantly lower in those with a *CBS* mutation. Adjustment for differences in vitamin concentrations, gender distribution, or the other amino acids strengthened the findings (Table 6).

The mutation in the majority (21 of 32) of mutated alleles was the pyridoxine-responsive R369C mutation *(30, 35)*. Also in samples with this mutation, methionine was high (g.mean = 23.4 μmol/L), whereas cystathionine (g.mean = 0.42 μmol/L) and CMR (g.mean = 1.91; $P = 0.016$) were low.

Using the random sample set, we investigated which of the variables best identified carriers of a *CBS* mutation. We defined abnormal concentrations as tHcy or methionine above the 90th percentile or cystathionine or CMR below the 10th percentile in the total population. The carrier state was observed in 1.7% of those with increased tHcy ($P > 0.05$), in 4.1% of those with increased methionine ($P > 0.05$), in 7.5% of those with low cystathionine ($P < 0.001$), and in 5.8% of those with low CMR ($P = 0.039$). Among those with a mutated *CBS* allele, 14.8% had increased methionine ($P > 0.05$), 7.1% had increased tHcy ($P > 0.05$), 29.6% had low cystathionine ($P < 0.001$), and 18.5% had low CMR ($P = 0.042$). ROC analyses confirmed that cystathionine and CMR significantly discriminated between heterozygosity and wild-type *CBS* ($P = 0.012$ and 0.001, respectively), whereas methionine and tHcy did not.

Changes in serum variables according to time of blood sampling relative to birth

In the Bergen set (n = 104), we had some data on sample handling. These samples did not differ significantly from the total population or the random sample set with respect to vitamin and metabolite concentrations or gender distribution.

The samples were categorized into three groups according to the time of blood collection: ≤3 days (n = 35), at day 4 (n = 47), and at days 5–8 (n = 22) after birth. After adjustment for unfrozen storage time and for gender, there was no significant change according to day of sampling for tHcy ($P = 0.45$), MMA ($P = 0.84$), B_{12} ($P = 0.70$), or CMR ($P = 0.45$). Methionine (g.mean = 20.4, 19.6, and 25.5 μmol/L; $P = 0.077$) and cystathionine (g.mean = 0.47, 0.49, and 0.68 μmol/L; $P = 0.054$) tended to increase. The difference in serum folate was highly significant ($P = 0.001$), being 23.9 nmol/L in the first group, 19.2 nmol/L in the second, and 14.1 nmol/L in the third group.

Changes in serum variables according to duration of unfrozen storage

Duration of unfrozen storage refers to the time from blood collection until serum and blood cells were transferred to the freezer. In this period, the sample was kept in a centrifuged gel separator tube, and most of the time it was kept refrigerated. The mean duration was 14 days (range, 8–29 days). We categorized the duration of unfrozen storage into three groups: ≤10 days (mean, 10 days; n = 22), 11–16 days (mean, 14 days; n = 63), and ≥17 days (mean, 20 days; n = 19). The metabolites, B_{12}, and CMR did not change significantly among the groups, but folate concentrations decreased as a function of unfrozen storage time. After adjustment for the day of sample collection relative to birth, g.mean serum folate was 23.6 nmol/L at a mean storage time of 10 days, 18.6 nmol/L at 14 days, and 16.3 nmol/L at 20 days ($P = 0.035$). Further adjustment for gender and differences in the other serum variables only marginally changed the findings.

Discussion

We have confirmed that low B_{12} status is a common cause of increased tHcy in newborn children *(13–15)*. An important new observation was that more marked tHcy increases usually were associated with increased methionine, which also caused increased cystathionine. We also observed that low cystathionine combined with increased methionine and tHcy was consistent with a mutated CBS allele, whereas increases in both cystathionine and tHcy combined with low or normal methionine indicated low B_{12} status or possibly another remethylation defect. Thus, our data suggest that measurement of methionine, cystathionine, and B_{12} are potentially useful for identifying probable causes of increased tHcy in newborns.

Study design

In this cross-sectional cohort using anonymous blood samples, it was only possible to study biomarkers that could be determined in a minute volume of blood. This reduced the number of variables measured, and lack of serum often prevented retesting of samples with results that were unexpected or outside the measurement range.

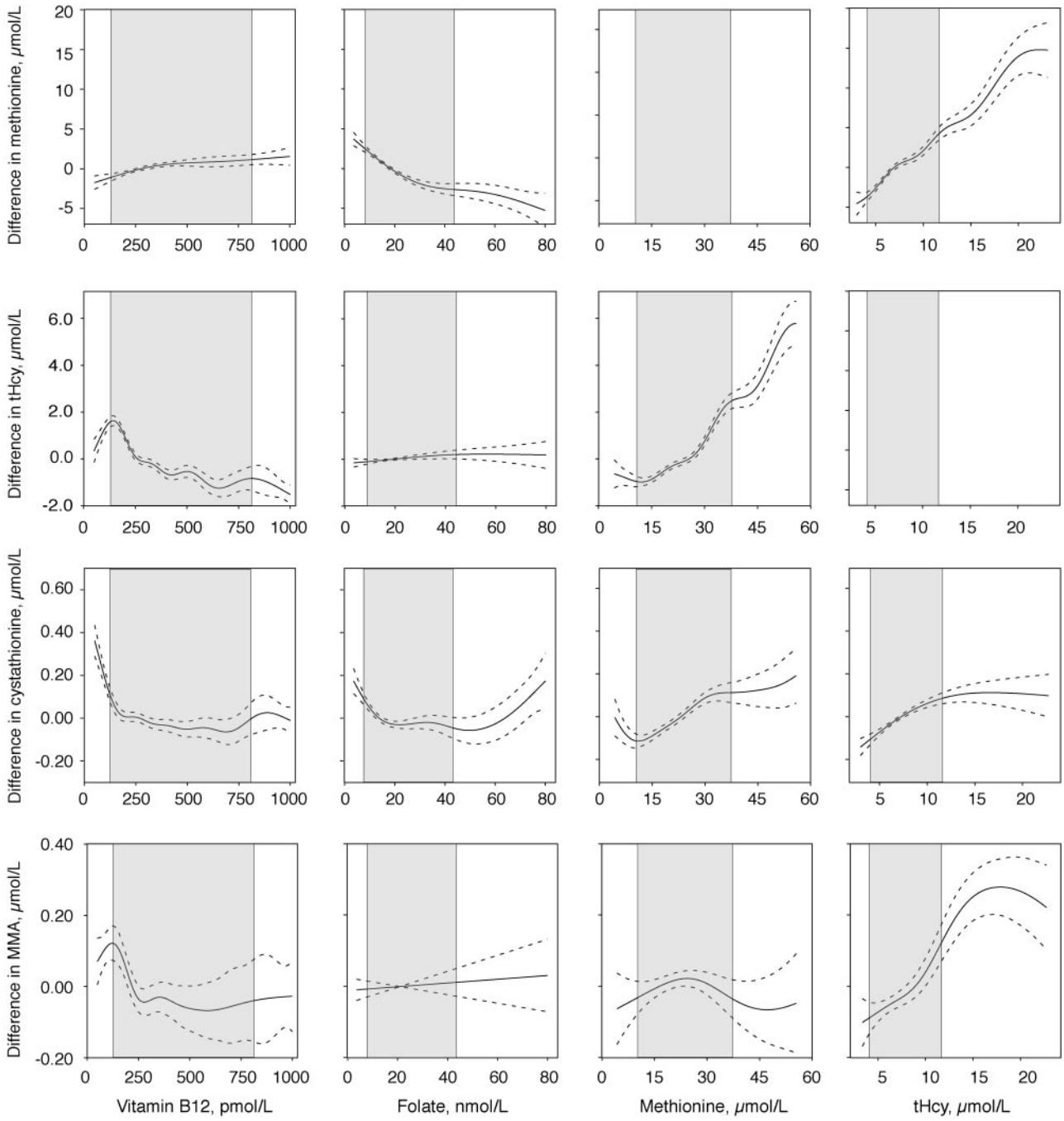

Fig. 2. Dose–response curves for the relationships between the serum variables.

The *curves* were obtained by use of gaussian-generalized additive models. Each *horizontal row* represents a specified regression model, with one panel for each independent variable (*x axis*) included in the model. The dependent (outcome) variable is indicated on the *y axis*. The *solid curve* in each panel is the estimated function, whereas the *dashed curves* are the approximate confidence lines (95% CI). *Shaded areas* are the 5th–95th percentile intervals (reference intervals) for the respective independent variables.

Another problem was that we had no clinical data, and we could therefore not examine the effects of other factors that may influence tHcy and B vitamin status in the newborn baby, including maternal B vitamin status *(14, 15)*, gestational age *(36, 37)*, breastfeeding vs other types of nutrient intake *(13)*, or the use of nitrous oxide during delivery *(38, 39)*. Although the metabolite profile points to the site of a defect, it rarely provides firm evidence of the underlying cause or the clinical consequences. In this regard, our data emphasize that a screening result is a complement to the clinical investigation and medical history of the child and his or her family.

Table 4. Serum variables (g.mean or proportion with abnormal results) according to categories of serum tHcy.[a,b]

Variable	Serum tHcy, μmol/L				
	<5	5–10	10–15	15–20	>20
n	745	3516	597	84	43
Percentage of population	14.9	70.5	12.0	1.69	0.86
Vitamin B_{12} (g.mean), pmol/L	395	304	236	228	258
Vitamin B_{12} <123 pmol/L, %	4.5	3.7[c]	10.9	12.2	8.1[c]
Folate (g.mean), nmol/L	18.7	17.6[d]	16.7	15.1	16.7[c]
Folate <7.5 nmol/L, %	4.1	4.7[c]	6.9[c]	8.4[c]	9.8[c]
Methionine (g.mean), μmol/L	17.4	20.4	23.7	29.7	35.8
Methionine >37.3 μmol/L, %	1.0	3.7	11.2	24.1	47.6
Cystathionine (g.mean), μmol/L	0.40	0.52	0.67	0.69	0.70
Cystathionine >1.24 μmol/L, %	3.1	4.1	9.1	10.1[d]	19.1
MMA (g.mean),[e] μmol/L	0.21	0.26	0.38	0.42	0.36
MMA >0.59 μmol/L,[e] %	0	4.1[c]	11.1	20.3	24.2

[a] Thresholds used to define abnormal values were the 5th percentile for the vitamins and the 95th percentile for the metabolites.

[b] Differences between categories were tested using ANOVA followed by post hoc test with Bonferroni correction. If not otherwise stated, the results are significantly different ($P < 0.05$) from the g.mean obtained in tHcy category <5 μmol/L.

[c] Not significantly different from g.mean obtained in tHcy category <5 μmol/L.

[d] P between 0.05 and 0.10 compared with tHcy <5 μmol/L.

[e] Based on the random sample set and samples with tHcy >15 μmol/L.

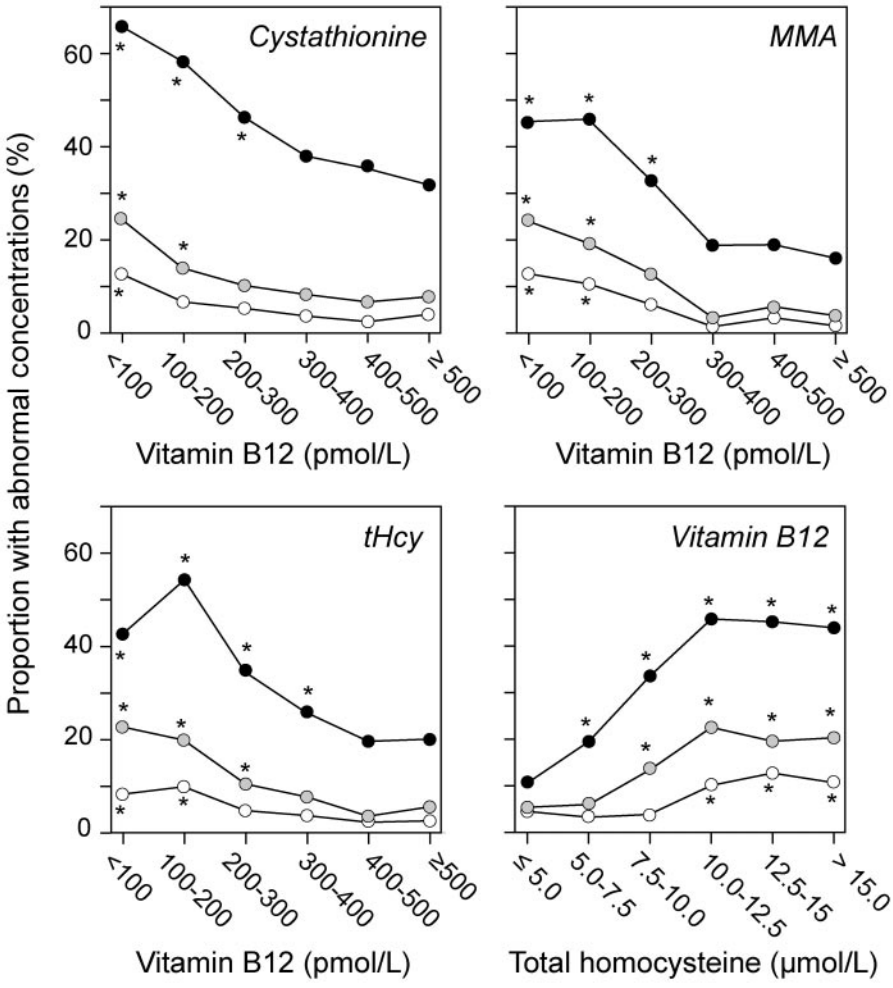

Fig. 3. Proportions of infants with increased metabolite concentrations according to serum B_{12} concentrations and with low serum B_{12} concentrations according to serum tHcy concentrations.

The thresholds to define abnormal concentrations were as follows: ○, 95th percentiles for metabolites and 5th percentile for B_{12}; ○, 90th percentiles for metabolites and 10th percentile for B_{12}; ●, values obtained by ROC analyses. Thresholds from ROC analyses were as follows: MMA = 0.30 μmol/L (73rd percentile); cystathionine = 0.55 μmol/L (58th percentile); tHcy = 8.0 μmol/L (68th percentile); and B_{12} = 220 pmol/L (25th percentile). *, significantly different from those with the highest B_{12} or lowest tHcy concentrations.

Table 5. Findings in samples with extremely high or low concentrations of the serum variables.

Extreme group[a]		g.mean[b] (range)					
		Methionine, μmol/L	tHcy, μmol/L	Cystathionine, μmol/L	Folate, nmol/L	Vitamin B_{12}, pmol/L	MMA, μmol/L
None (total population)		20.4	7.1	0.51	17.8	302	0.26
Methionine	Highest (n = 11)	**69.2 (62.8–115.1)**[c]	**10.0**	**1.02**	**10.5**	251	0.29[d]
	Lowest (n = 10)	**1.4 (0.6–2.5)**[c]	7.6	**0.91**	**49.0**	389	0.26[d]
tHcy	Highest (n = 10)	**41.7**	**30.2 (28.4–36.8)**[c]	**0.74**	12.9[e]	263	0.31
	Lowest (n = 10)	**11.0**	**2.5 (1.9–2.8)**[c]	0.39	21.4	331	0.25[d]
Cystathionine	Highest (n = 10)	22.9	7.6	**5.89 (4.04–10.34)**[c]	14.5	162	–
	Lowest (n = 10)	**7.2**	**5.4**	**0.08 (0.02–0.11)**[c]	23.4	417	0.22[d]
Folate	Highest (n = 25)	**10.2**	6.8	0.55	**>80 (>80)**[c]	**380**	0.25
	Lowest (n = 15)	19.5	7.6	0.46	**<2.0 (<2.0)**[c]	240	0.33[d]
Vitamin B_{12}	Highest (n = 162)	19.5	**5.6**	**0.44**	**20.9**	**>1000 (>1000)**[c]	**0.22**
	Lowest (n = 79)	19.1	**6.2**	**0.65**	**14.8**	**<50 (<50)**[c]	0.27
MMA	Highest (n = 11)	22.4	**11.5**	**0.83**	21.4	**170**	**2.04 (1.35–4.89)**[c]
	Lowest (n = 13)	**11.2**	**5.2**	**0.33**	**26.9**	347	**0.11 (0.09–0.12)**[c]

[a] The extreme group consisted of the 10–13 highest or lowest values (0.2th percentile for the amino acids, and 1.4th percentile for MMA). For vitamins with restricted measurement ranges, the extreme groups included more values.

[b] Bolded numbers indicate a g.mean that is different from the g.mean of the total population (Student *t*-test and confirmed with Mann–Whitney *U*-test).

[c] Results of the extreme group for the selected variable.

[d] Include fewer than four values, and statistical difference was not tested.

[e] $P = 0.06$.

Table 6. Geometric mean tHcy, methionine, and cystathionine concentrations according to heterozygosity for *CBS* mutations.

	Unadjusted			Adjusted[a]		
	CBS			*CBS*		
	Wild type	Heterozygous	*P*[b]	Wild type	Heterozygous	*P*[c]
Random sample set						
n	1102	28		723	18	
Methionine, μmol/L	19.4	22.2	0.095	19.3	23.6	0.047
tHcy, μmol/L	7.0	6.4	0.16	7.0	6.7	0.56
Cystathionine, μmol/L	0.52	0.42	0.028	0.52	0.41	0.022
CMR	2.68	1.89	0.002	2.72	1.82	0.004
tHcy >15 μmol/L and/or methionine >40 μmol/L						
n	242	6		225	6	
Methionine, μmol/L	38.6	50.5	0.045	38.8	50.9	0.019
tHcy, μmol/L	12.8	12.4	0.87	12.4	14.5	0.39
Cystathionine, μmol/L	0.71	0.46	0.062	0.71	0.46	0.040
CMR	1.85	0.92	0.006	1.84	0.98	0.006

[a] Each of the amino acids was adjusted for gender, B_{12}, and the two other amino acids. The CMR was adjusted for gender, B_{12}, and tHcy.
[b] Student *t*-test.
[c] Analysis of covariance.

analyte stability in unfrozen samples

The samples in this study were kept unfrozen for ~14 days, which could affect concentrations of metabolites or vitamins. However, the concentrations of methionine, tHcy, MMA, and B_{12} were similar to those found in a screening population *(40)* or smaller studies of newborns *(13, 14)*. These variables also seemed stable during unfrozen storage. In contrast, we observed that serum folate was lower than in optimally handled samples *(13, 14)*. This is consistent with our findings that folate decreased according to time before freezing and previous data that folate is not stable in unfrozen samples *(41–43)*. Although the sample handling may have weakened the associations, it is unlikely to have created false associations.

changes in vitamins and metabolites after birth

Data on the changes in metabolites and vitamin in early infancy are sparse. In the first weeks after birth, plasma concentrations of tHcy and folate are relatively stable *(17)*, whereas MMA increases dramatically *(14)*, sometimes leading to a benign or transient methylmalonic acidemia *(44)*. The changes in MMA are only partly explained by changes in B_{12} concentrations *(14)*. Data on changes during the first few days of life in full-term infants are not available, but in newborn premature infants, tHcy tends to increase, probably as a result of parenteral nutrition *(36)*, and in preterm baboons, methionine, cystathionine, and tHcy increase *(45)*. In the babies for whom we knew the time of sampling, we found that B_{12}, MMA, and tHcy did not differ according to time since birth, whereas serum folate decreased markedly and methionine and cystathionine tended to increase. Such changes may lead to confounding in studies in which the time of sampling differs among the babies. In the clinical setting, it has implications for reference intervals. Our findings should therefore be confirmed in more optimally designed studies.

gender differences

A gender difference in B_{12} concentrations in newborns has previously not been reported. MMA is, however, higher in newborn girls than in boys but not later in infancy *(17)*. In the present study, we found that all factors associated with serum B_{12} were consistent with a better B_{12} status in boys than in girls. There are gender differences in the B_{12} binding proteins in the amniotic fluid *(46)*, and it is therefore possible that the differences between girls and boys have developed in utero. The clinical implication of this finding is uncertain.

folate, *MTHFR* genotypes, and tHcy

In children and adults, serum folate is a strong determinant of tHcy concentrations *(17, 47)*. Some *(13, 15)*, but not all studies *(14)*, have also found a significant association in newborns. The generally high folate concentrations in infants may explain the relatively weak tHcy–folate relationship *(17)*. The *MTHFR* 677C>T polymorphism is a strong determinant of tHcy in older children and adults *(48–50)*, but not in children <10 years *(48)*. Our data show that tHcy was not associated with serum folate or with the 677C>T polymorphism.

Usually, the association between the *MTHFR* 677C>T genotypes and tHcy is more pronounced in those with low folate concentrations. However, even in babies with serum folate <5 nmol/L (approximately the 1st percentile), the TT genotype had no effect on tHcy. It has been suggested that high serum folate during infancy is attrib-

utable to low B_{12} status and the resulting methyl folate trap phenomenon *(16, 17)*. Another, and more likely, cause is that the supply of folate is high via the placenta *(51)* and in breast milk *(52, 53)*. In line with this, tissue uptake of folate is increased during infancy *(54)*, and in infants, erythrocyte folate is higher than later in childhood *(17)*. Although folate is a vital nutrient for the fetus and growing infant, low folate concentrations are not a common cause of increased tHcy in newborns.

VITAMIN B_{12} STATUS

Our data confirm that many babies have low B_{12} concentrations *(13–15)*. We found that ~10% had B_{12} under the adult lower reference limit of 150 pmol/L. These babies frequently had increased tHcy, cystathionine, and/or MMA as well. Use of ROC analyses to test the ability of tHcy, cystathionine, MMA, and B_{12} to separate between those with or without biochemical disturbances of B_{12} status revealed that all four variables had approximately equal discriminatory power.

Published results on B_{12} and cystathionine in screening samples are lacking, and data on tHcy are sparse *(20)*. Screening of MMA sometimes takes place with the intention of discovering inborn errors *(2)*. Urine screening carried out some weeks after birth has shown that most babies with methylmalonic aciduria have transient or benign variants of the condition *(55)*. MS/MS analysis of blood spots from newborns revealed that MMA is increased in 1 in 150 000. Approximately 30% of these have B_{12} deficiency *(55)*. Our data, as well as results from another study *(14)*, suggest that the less extreme MMA concentrations are also strongly associated with low B_{12} and increased tHcy concentrations.

Newborn screening for B_{12} status would differ from the typical metabolic screening, in which fewer than 1 in 1000 babies undergo further investigation *(40)*. On the basis of the thresholds we found by ROC analyses, at least 25% of babies would be considered B_{12}-deficient. More restrictive thresholds combined with a second test could improve diagnostic accuracy and reduce the prevalence. Indeed, diagnosis of B_{12} deficiency or low B_{12} status should depend on at least two findings: low B_{12} combined with clinical symptoms, or low B_{12} combined with increased metabolites *(47)*. Use of a combination of tHcy >10 μmol/L and B_{12} <200 pmol/L *(47)* or tHcy >10 μmol/L and MMA >0.40 μmol/L indicated that ~5% of babies have biochemical evidence of impaired B_{12} function. These babies may be at risk of developing a clinical deficiency later in infancy, particularly if the baby is exclusively breastfed. The fact that the condition is common and can easily be prevented, treated, and cured is no valid argument against routine testing *(2)*. On the contrary, a diagnosis of B_{12} deficiency in a baby, which is usually caused by low B_{12} status in the mother, is unlikely to cause the same parental stress and anxiety as a diagnosis of an inborn error *(56)*.

Currently, we lack clinical practice guidelines for diagnosis and prevention of B_{12} deficiency in young children *(19)*. Measurement of tHcy, B_{12}, or other biomarkers in the newborn baby and/or mother is only one possibility. A more proactive approach is to advise vegetarians or perhaps all mothers to use B_{12} supplements during pregnancy and lactation or to give a B_{12} supplement to the baby while breastfeeding *(19)*. Yet another possibility is to introduce fortification of B_{12} in flour *(57)*. To identify the optimal approach will require more studies with clinical as well as laboratory data. In particular, it would be useful to carry out longitudinal studies in high-risk groups such as babies of vegetarian mothers.

CBS DEFICIENCY

The typical metabolic pattern in CBS deficiency is increased methionine combined with severely increased tHcy (>100 μmol/L) and very low cystathionine concentrations (<0.10 μmol/L) *(8, 58)*. The carrier state is associated with few or no symptoms *(3)*, and tHcy is within the reference interval *(3, 58)*, whereas cystathionine tends to be low *(58)*.

In our study, we found no babies with the typical metabolic pattern of CBS deficiency, and analysis for selected mutations in those with increased methionine and/or tHcy further support that none of the babies had homocystinuria. The prevalence of heterozygosity for CBS deficiency was 2.47% *(6)*. In these babies, cystathionine was low and methionine tended to be high, whereas tHcy was within the reference interval, i.e., a pattern similar to that observed in heterozygous adults *(3, 58)*.

Low cystathionine was the best marker among the three amino acids for detecting carriers of a defective *CBS* allele, and this confirms that cystathionine is particularly useful in the assessment of CBS function *(59)*. Low cystathionine is, however, not a specific finding. In our study, many samples with low cystathionine had low tHcy and methionine as well. This could be measurement error because all three amino acids were determined in the same run. Cystathionine is also very sensitive to changes in methionine intake *(60)*, and in the babies, cystathionine was strongly associated with methionine concentration. We found that use of CMR circumvented a potential measurement error and reduced the impact of changes in methionine concentrations on cystathionine.

Because we did not find any baby with CBS deficiency, we can only speculate about the optimum approach for identification of this condition. Current screening programs, based on methionine measurement alone, often miss pyridoxine-responsive variants of CBS deficiency *(3)*. Our data suggest that even a "mild" and very pyridoxine-responsive mutation such as the R369C mutation *(30, 35)* is associated with a metabolic pattern consistent with CBS impairment. This is promising in relation to homocystinuria screening, and we therefore suggest that tHcy and cystathionine should be further evaluated and compared with methionine in their ability, on their own or jointly, to detect CBS deficiency.

other potential causes of extremely increased methionine

A problem with the current screening test for CBS deficiency, methionine determination, is the high rate of false-positive results *(2, 61)*. In our population, none of the babies with the most extreme increased methionine concentrations had mutated *CBS* alleles or an amino acid pattern suggesting homocystinuria. In newborns and infants, food formulas and amino acid solutions may cause hypermethioninemia and hyperhomocysteinemia *(3, 62)*, and severe hypermethioninemia is often found in babies who are premature, have low birth weights, or who are in neonatal intensive care units *(40, 62)*. Deficiencies of methionine adenosyltransferase I/III *(59)* and glycine N-methyltransferase *(63)* may also cause extreme increases in methionine, to the same concentrations observed in CBS deficiency. tHcy may be moderately increased. However, these inborn errors as well as intake of excess methionine are associated with increased cystathionine *(59, 63)*, whereas cystathionine in CBS deficiency always is very low *(58, 59)*.

relationship between methionine and folate

An unexpected finding in our study was the inverse association between folate and methionine. This could be related to our observation that folate decreases whereas methionine increases in the first few days after birth. Another possibility is that low methionine, via *S*-adenosylmethionine, stimulates MTHFR activity and enhances formation of 5-methyltetrahydrofolate *(64)*, which we detected as increased serum folate. The reason for the finding of low methionine is not clear. B_{12} deficiency could be one explanation *(64)*, but in samples with extremely low methionine (which consistently had increased folate), B_{12} was in the high range and tHcy was within the reference interval. Nitrous oxide exposure, which inactivates methionine synthase and thereby leads to increased serum folate, low methionine, and increased tHcy *(65)*, is yet another possibility. However, at least in adults, the nitrous oxide effect on folate and methionine concentrations is short, whereas the effect on tHcy persists for many days *(65, 66)*. Thus, the findings in these babies are not consistent with nitrous oxide exposure. A third possibility is a primary methionine deficiency *(64)*. Methionine is low in preterm infants before they receive amino acid supplementation *(67)* and may be low in infants before they start nursing. However, the high cystathionine suggests that homocysteine was directed toward transsulfuration at a time when methionine was needed. This points to a remethylation defect. MTHFR, methionine synthase, and betaine homocysteine methyltransferase are fully active at birth *(45, 68)*, and the latter is usually up-regulated under conditions of low methionine *(69)*. Measurement of factors associated with betaine-dependent remethylation, such as betaine and dimethylglycine, may provide important clues. If remethylation defects lead to methionine deficiency, which in turn leads to low tHcy, this may have implications for the use of tHcy measurement in the detection of homocystinuria attributable to remethylation defects.

very high cystathionine concentrations

In our cohort, four babies had markedly increased cystathionine >6 μmol/L; all of them had tHcy <10 μmol/L and three of four had methionine <20 μmol/L. Such a pattern is found in cystathioninuria *(59)*, which is an autosomal recessive disorder without certain clinical consequences *(3)*. A more likely cause is related to cystathionine lyase activity, which is low at birth, particularly in premature babies *(3, 70)*. Increased cystathionine is also observed in deficiency of vitamin B_6, which has a much stronger effect on cystathionine lyase than on CBS *(3)*. In line with this, cystathioninuria in premature infants often responds to vitamin B_6 treatment *(71)*. The possible significance of vitamin B_6 status in newborns should be further investigated.

very low B_{12} concentrations

A most surprising finding was that babies with the lowest B_{12} concentrations (<50 pmol/L; n = 75) had completely normal MMA and significantly lower tHcy concentrations than the remainder of the population. In contrast, cystathionine was increased and, thus, consistent with impaired B_{12} function. The explanation for the low B_{12} is not clear. It could be attributable to measurement error or that the samples were from babies who were treated with antibiotics or agents that interfere with the B_{12} assay. An alternative explanation is that the active form of B_{12} in serum, holotranscobalamin, is normal, whereas B_{12} bound to the nonactive haptocorrins is low or deficient *(72)*. However, none of these possibilities seem to fully explain the observed pattern. Whatever the reason, the metabolic consequences seem mild, and the normal methionine suggests that the babies are protected from the serious sequalae of B_{12} deficiency *(73)*.

algorithm for using tHcy measurements in newborns

On the basis of published data and the findings in this study, we suggest an algorithm for using tHcy measurements in newborn screening (Fig. 4). The evidence for routinely using tHcy in newborns is weak. Although it is possible, perhaps even probable, it remains to be shown that tHcy actually is better than methionine for the detection of all types of homocystinuria, including pyridoxine-responsive variants and remethylation defects. Moreover, we do not know that identifying and treating a newborn baby with low B_{12} will prevent serious complications. However, tHcy is increasingly being measured in newborns, and even without sufficient evidence, one needs guidelines for its use *(47)*. In our opinion, it is reasonable to routinely test babies who are at high risk of B_{12} deficiency (e.g., if the mother is a vegetarian) and babies with close family members with homocystinuria.

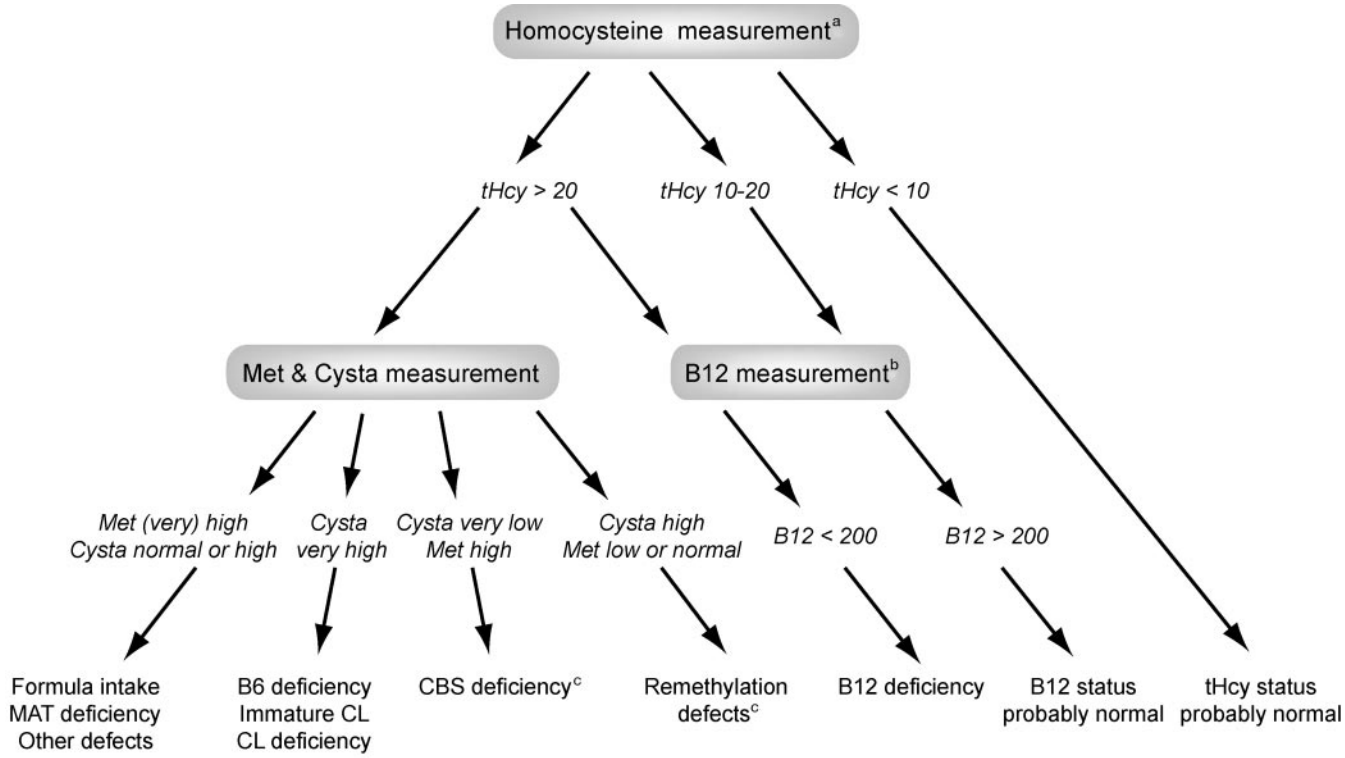

Fig. 4. Algorithm for using tHcy measurements in newborns at high risk of B_{12} deficiency or who have a close family member with homocystinuria.

[a] For several of the metabolic disorders shown, tHcy is not the optimum primary test. [b] Instead of B_{12}, it is possible to perform MMA measurements, using a threshold of ~0.40 μmol/L. Later in infancy, MMA is less useful because it is not correlated to B_{12} status *(14)*. [c] In CBS deficiency and in remethylation defects, the use of CMR and the ratio between tHcy and methionine may partly circumvent the effect related to changes in methionine and tHcy concentrations. *Cysta*, cystathionine; *MAT*, methionine adenosyltransferase I/III; *CL*, cystathionine γ-lyase.

In conclusion, our study brings the use of tHcy measurement in newborns a step forward but not to a stage where we can recommend routine screening of all babies. An obvious limitation with the current study is that we had no clinical data on the babies, nor did we have a defined protocol for sample collection and handling. However, this is typical for the screening situation, and despite such limitations, our study demonstrates how multiple measurements in a single blood sample can reveal probable causes of hyperhomocysteinemia. In newborn-screening programs, further investigation and follow-up are usually confined to babies with the most extreme concentrations of a single analyte, based on the assumption that extreme values reflect the seriousness of a disease. This policy is also determined by the wish to keep false-positive results, and the risk of parental anxiety, at an acceptable level. Our data suggest that one may get a more accurate diagnosis by investigating more of the babies and by use of a more varied set of analyses. With the advent of new technologies, in particular MS/MS, the one blood sample provided by the routine screening could often be all that is needed.

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Contributions

H. Refsum: concept, design, collection and analyses of data and preparation of the manuscript.
A.W. Grindflek: design, collection of samples.
P.M. Ueland: design and critical revision of the manuscript.
Å. Fredriksen: optimizing the mass spectrometry method and performing the *CBS* mutation analysis.
K. Meyer: optimizing the mass spectrometry method and performing the *CBS* mutation analysis.
A. Ulvik: optimizing the real-time PCR methods and performing the *SRY* and *MTHFR* gene analyses.

A.B. Guttormsen: concept and optimization of the MMA method.
O.E. Iversen: collection of samples and data related to the Bergen set.
J. Schneede: critical revision of the manuscript.
B.F. Kase: concept, design, collection of data, and critical revision of the manuscript.

References

1. Serving the family from birth to the medical home. Newborn screening: a blueprint for the future—a call for a national agenda on state newborn screening programs. Pediatrics 2000;106:389–422.
2. Fearing MK, Levy HL. Expanded newborn screening using tandem mass spectrometry. Adv Pediatr 2003;50:81–111.
3. Mudd SH, Levy HL, Skovby F. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease, 7th ed. New York: McGraw-Hill, 1995:1279–327.
4. Gaustadnes M, Ingerslev J, Rutiger N. Prevalence of congenital homocystinuria in Denmark. N Engl J Med 1999;340:1513.
5. Linnebank M, Homberger A, Junker R, Nowak-Goettl U, Harms E, Koch HG. High prevalence of the I278T mutation of the human cystathionine β-synthase detected by a novel screening application. Thromb Haemost 2001;85:986–8.
6. Refsum H, Fredriksen A, Meyer K, Ueland PM, Kase BF. Birth prevalence of homocystinuria. J Pediatr 2004;144:830–2.
7. Moat SJ, Bao L, Fowler B, Bonham JR, Walter JH, Kraus JP. The molecular basis of cystathionine β-synthase (CBS) deficiency in UK and US patients with homocystinuria. Hum Mutat 2004;23:206.
8. Orendac M, Zeman J, Stabler SP, Allen RH, Kraus JP, Bodamer O, et al. Homocystinuria due to cystathionine β-synthase deficiency: novel biochemical findings and treatment efficacy. J Inherit Metab Dis 2003;26:761–73.
9. Yap S, Boers GH, Wilcken B, Wilcken DE, Brenton DP, Lee PJ, et al. Vascular outcome in patients with homocystinuria due to cystathionine β-synthase deficiency treated chronically: a multicenter observational study. Arterioscler Thromb Vasc Biol 2001;21:2080–5.
10. Naughten ER, Yap S, Mayne PD. Newborn screening for homocystinuria: Irish and world experience. Eur J Pediatr 1998;157(Suppl 2):S84–7.
11. Rosenblatt DS, Whitehead VM. Cobalamin and folate deficiency: acquired and hereditary disorders in children. Semin Hematol 1999;36:19–34.
12. Stabler SP, Lindenbaum J, Savage DG, Allen RH. Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. Blood 1993;81:3404–13.
13. Minet JC, Bisse E, Aebischer CP, Beil A, Wieland H, Lutschg J. Assessment of vitamin B-12, folate, and vitamin B-6 status and relation to sulfur amino acid metabolism in neonates. Am J Clin Nutr 2000;72:751–7.
14. Bjorke Monsen AL, Ueland PM, Vollset SE, Guttormsen AB, Markestad T, Solheim E, et al. Determinants of cobalamin status in newborns. Pediatrics 2001;108:624–30.
15. Guerra-Shinohara EM, Paiva AA, Rondo PH, Yamasaki K, Terzi CA, D'Almeida V. Relationship between total homocysteine and folate levels in pregnant women and their newborn babies according to maternal serum levels of vitamin B_{12}. BJOG 2002;109:784–91.
16. Ueland PM, Monsen AL. Hyperhomocysteinemia and B-vitamin deficiencies in infants and children. Clin Chem Lab Med 2003;41:1418–26.
17. Monsen AL, Refsum H, Markestad T, Ueland PM. Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years. Clin Chem 2003;49:2067–75.
18. Fokkema MR, Woltil HA, van Beusekom CM, Schaafsma A, Dijck-Brouwer DA, Muskiet FA. Plasma total homocysteine increases from day 20 to 40 in breastfed but not formula-fed low-birthweight infants. Acta Paediatr 2002;91:507–11.
19. From the Centers for Disease Control and Prevention. Neurologic impairment in children associated with maternal dietary deficiency of cobalamin—Georgia, 2001. JAMA 2003;289:979–80.
20. Accinni R, Campolo J, Parolini M, De Maria R, Caruso R, Maiorana A, et al. Newborn screening of homocystinuria: quantitative analysis of total homocyst(e)ine on dried blood spot by liquid chromatography with fluorimetric detection. J Chromatogr B Analyt Technol Biomed Life Sci 2003;785:219–26.
21. Husek P. Simultaneous profile analysis of plasma amino and organic acids by capillary gas chromatography. J Chromatogr B Biomed Appl 1995;669:352–7.
22. Frantzen F, Faaren AL, Alfheim I, Nordhei AK. Enzyme conversion immunoassay for determining total homocysteine in plasma or serum. Clin Chem 1998;44:311–6.
23. Nexo E, Engbaek F, Ueland PM, Westby C, O'Gorman P, Johnston C, et al. Evaluation of novel assays in clinical chemistry: quantification of plasma total homocysteine. Clin Chem 2000;46:1150–6.
24. Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8:135–60.
25. O'Broin S, Kelleher B. Microbiological assay on microtitre plates of folate in serum and red cells. J Clin Pathol 1992;45:344–7.
26. Kelleher BP, Walshe KG, Scott JM, O'Broin SD. Microbiological assay for vitamin B_{12} with use of a colistin-sulfate-resistant organism. Clin Chem 1987;33:52–4.
27. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997;281:43–53.
28. Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. First-trimester fetal sex determination in maternal serum using real-time PCR. Prenat Diagn 2001;21:1070–4.
29. Ulvik A, Ueland PM. Single nucleotide polymorphism (SNP) genotyping in unprocessed whole blood and serum by real-time PCR: application to SNPs affecting homocysteine and folate metabolism. Clin Chem 2001;47:2050–3.
30. Kim CE, Gallagher PM, Guttormsen AB, Refsum H, Ueland PM, Ose L, et al. Functional modeling of vitamin responsiveness in yeast: a common pyridoxine-responsive cystathionine β-synthase mutation in homocystinuria. Hum Mol Genet 1997;6:2213–21.
31. Harksen A, Ueland PM, Refsum H, Meyer K. Four common mutations of the cystathionine β-synthase gene detected by multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem 1999;45:1157–61.
32. Hastie T, Tibshirani R. Generalized additive models for medical research. Stat Methods Med Res 1995;4:187–96.
33. R-Development-Core-Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2004. http://www.R-project.org (accessed April 2004).
34. Walter SD. Properties of the summary receiver operating characteristic (SROC) curve for diagnostic test data. Stat Med 2002;21:1237–56.
35. Gaustadnes M, Wilcken B, Oliveriusova J, McGill J, Fletcher J, Kraus JP, et al. The molecular basis of cystathionine β-synthase deficiency in Australian patients: genotype-phenotype correlations and response to treatment. Hum Mutat 2002;20:117–26.
36. Hongsprabhas P, Saboohi F, Aranda JV, Bardin CL, Kovacs LB, Papageorgiou AN, et al. Plasma homocysteine concentrations of preterm infants. Biol Neonate 1999;76:65–71.

37. Febriani AD, Sakamoto A, Ono H, Sakura N, Ueda K, Yoshii C, et al. Determination of total homocysteine in dried blood spots using high performance liquid chromatography for homocystinuria newborn screening. Pediatr Int 2004;46:5–9.
38. Landon MJ, Toothill VJ. Effect of nitrous oxide on placental methionine synthase activity. Br J Anaesth 1986;58:524–7.
39. Zanardo V, Caroni G, Burlina A. Higher homocysteine concentrations in women undergoing caesarean section under general anesthesia. Thromb Res 2003;112:33–6.
40. Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, et al. Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin Chem 2001;47:1945–55.
41. O'Broin JD, Scott JM, Temperley IJ. A comparison of serum folate estimations using two different methods. J Clin Pathol 1973;26: 80–1.
42. Lawrence JM, Umekubo MA, Chiu V, Petitti DB. Split sample analysis of serum folate levels after 18 days in frozen storage. Clin Lab 2000;46:483–6.
43. Mastropaolo W, Wilson MA. Effect of light on serum B_{12} and folate stability. Clin Chem 1993;39:913.
44. Sniderman LC, Lambert M, Giguere R, Auray-Blais C, Lemieux B, Laframboise R, et al. Outcome of individuals with low-moderate methylmalonic aciduria detected through a neonatal screening program. J Pediatr 1999;134:675–80.
45. Stabler SP, Morton RL, Winski SL, Allen RH, White CW. Effects of parenteral cysteine and glutathione feeding in a baboon model of severe prematurity. Am J Clin Nutr 2000;72:1548–57.
46. Gimsing P, Toft L, Felbo M, Hippe E. Vitamin B_{12} binding proteins in amniotic fluid. Acta Obstet Gynecol Scand 1985;64:121–6.
47. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem 2004;50:3–32.
48. Delvin EE, Rozen R, Merouani A, Genest J Jr, Lambert M. Influence of methylenetetrahydrofolate reductase genotype, age, vitamin B-12, and folate status on plasma homocysteine in children. Am J Clin Nutr 2000;72:1469–73.
49. Tonstad S, Refsum H, Ose L, Ueland PM. The C677T mutation in the methylenetetrahydrofolate reductase gene predisposes to hyperhomocysteinemia in children with familial hypercholesterolemia treated with cholestyramine. J Pediatr 1997;132:365–8.
50. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996;93:7–9.
51. Giugliani ER, Jorge SM, Goncalves AL. Serum and red blood cell folate levels in parturients, in the intervillous space of the placenta and in full-term newborns. J Perinat Med 1985;13:55–9.
52. Mackey AD, Picciano MF. Maternal folate status during extended lactation and the effect of supplemental folic acid. Am J Clin Nutr 1999;69:285–92.
53. Smith AM, Picciano MF, Deering RH. Folate intake and blood concentrations of term infants. Am J Clin Nutr 1985;41:590–8.
54. Shojania AM, Hornady G. Folate metabolism in newborns and during early infancy. II. Clearance of folic acid in plasma and excretion of folic acid in urine by newborns. Pediatr Res 1970;4: 422–6.
55. Leonard JV, Vijayaraghavan S, Walter JH. The impact of screening for propionic and methylmalonic acidaemia. Eur J Pediatr 2003; 162(Suppl 1):S21–4.
56. Waisbren SE, Albers S, Amato S, Ampola M, Brewster TG, Demmer L, et al. Effect of expanded newborn screening for biochemical genetic disorders on child outcomes and parental stress. JAMA 2003;290:2564–72.
57. Oakley GP Jr. Let's increase folic acid fortification and include vitamin B-12. Am J Clin Nutr 1997;65:1889–90.
58. Guttormsen AB, Ueland PM, Kruger WD, Kim CE, Ose L, Folling I, et al. Disposition of homocysteine in subjects heterozygous for homocystinuria due to cystathionine β-synthase deficiency: relationship between genotype and phenotype. Am J Med Genet 2001;100:204–13.
59. Stabler SP, Steegborn C, Wahl MC, Oliveriusova J, Kraus JP, Allen RH, et al. Elevated plasma total homocysteine in severe methionine adenosyltransferase I/III deficiency. Metabolism 2002;51: 981–8.
60. Guttormsen AB, Solheim E, Refsum H. Variation in plasma cystathionine and its relation to changes in plasma concentrations of homocysteine and methionine in healthy subjects during a 24-h observation period. Am J Clin Nutr 2004;79:76–9.
61. Peterschmitt MJ, Simmons JR, Levy HL. Reduction of false negative results in screening of newborns for homocystinuria. N Engl J Med 1999;341:1572–6.
62. Mudd SH, Braverman N, Pomper M, Tezcan K, Kronick J, Jayakar P, et al. Infantile hypermethioninemia and hyperhomocysteinemia due to high methionine intake: a diagnostic trap. Mol Genet Metab 2003;79:6–16.
63. Mudd SH, Cerone R, Schiaffino MC, Fantasia AR, Minniti G, Caruso U, et al. Glycine *N*-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninaemia. J Inherit Metab Dis 2001;24:448–64.
64. Scott JM, Weir DG. The methyl folate trap. A physiological response in man to prevent methyl group deficiency in kwashiorkor (methionine deficiency) and an explanation for folic-acid induced exacerbation of subacute combined degeneration in pernicious anaemia. Lancet 1981;2:337–40.
65. Ermens AA, Refsum H, Rupreht J, Spijkers LJ, Guttormsen AB, Lindemans J, et al. Monitoring cobalamin inactivation during nitrous oxide anesthesia by determination of homocysteine and folate in plasma and urine. Clin Pharmacol Ther 1991;49:385–93.
66. Christensen B, Guttormsen AB, Schneede J, Riedel B, Refsum H, Svardal A, et al. Preoperative methionine loading enhances restoration of the cobalamin-dependent enzyme methionine synthase after nitrous oxide anesthesia. Anesthesiology 1994;80:1046–56.
67. Van Goudoever JB, Colen T, Wattimena JL, Huijmans JG, Carnielli VP, Sauer PJ. Immediate commencement of amino acid supplementation in preterm infants: effect on serum amino acid concentrations and protein kinetics on the first day of life. J Pediatr 1995;127:458–65.
68. Kalnitsky A, Rosenblatt D, Zlotkin S. Differences in liver folate enzyme patterns in premature and full term infants. Pediatr Res 1982;16:628–31.
69. Park EI, Garrow TA. Interaction between dietary methionine and methyl donor intake on rat liver betaine-homocysteine methyltransferase gene expression and organization of the human gene. J Biol Chem 1999;274:7816–24.
70. Zlotkin SH, Anderson GH. The development of cystathionase activity during the first year of life. Pediatr Res 1982;16:65–8.
71. Endres W, Vogt R, Riegel KP, Bremer HJ. The influence of vitamin B-6 on cystathioninuria in premature infants. Clin Chim Acta 1978;86:89–93.
72. Carmel R. R-binder deficiency. A clinically benign cause of cobalamin pseudodeficiency. JAMA 1983;250:1886–90.
73. Scott JM, Dinn JJ, Wilson P, Weir DG. Pathogenesis of subacute combined degeneration: a result of methyl group deficiency. Lancet 1981;2:334–7.