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 B12 (B12) 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 B12 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), B12 <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. B12 status, as determined by serum concentrations of B12, tHcy, and MMA, was moderately better in boys than in girls. tHcy concentrations between 10 and 20 μmol/L were often associated with low B12, 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 B12 deficiency.

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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 homocysteine in urine and total homocysteine (tHcy) and methionine in blood.

* Nonstandard abbreviations: CBS, cystathionine β-synthase; tHcy, total homocysteine; MTHFR, 5,10-methylenetetrahydrofolate reductase; B12, vitamin B12 (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).
(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 B₁₂ (B₁₂) 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 B₁₂, 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 B₁₂ concentrations (13–15). Such neonatal B₁₂ deficiency is nearly always attributable to low maternal B₁₂ status (16). These babies already have low B₁₂ combined with increased concentrations of tHcy and the specific B₁₂ marker, methylmalonic acid (MMA; Fig. 1) at birth. They continue to have a low B₁₂ status during infancy, particularly if the baby is exclusively breastfed (13, 17, 18). B₁₂ deficiency may lead to failure to thrive and developmental delay in infancy and lower cognitive function later in childhood (16). Low B₁₂ status is usually easily corrected by supplying vitamin B₁₂ (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 B₁₂ 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 B₁₂ 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 erythrocyte and serum was 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 B₁₂ 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 B₁₂ or increased tHcy or methionine.** The random sets had <25 samples with B₁₂ <100 pmol/L, tHcy >15 μmol/L, or methionine >40 μmol/L. To assess the biochemical relationships at these parts of the distribution, we measured MMA in all samples with B₁₂ <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, B₁₂ <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 [d₃,l-methionine-3,3,4,4-d₄; d₃,l-homocysteine-3,3',3',4,4,4'-d₄; and d₃,l-(2-amino-2-carboxyethyl)-homocysteine-3,3,4,4-d₄ (cystathionine-d₄)], the sample was acid-precipitated, and the supernatant was injected on a C₁₈ 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-d₄, m/z 140→94; methionine-d₄, m/z 154→108; cystathionine-d₄, 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: tHcyEIA – tHcyLC-MS/MS = 0.02(tHcy - mean) – 0.17; R = 0.03. Thus, pooled data are presented.

**Folate and B₁₂ determination.** Serum folate and B₁₂ 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 B₁₂ assay measures total B₁₂ 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 B₁₂ assays were adapted to a microtiter plate format (27), and carried out by a robotic workstation. The measurement range for B₁₂ 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 χ² 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 B12 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 B12 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, B12, and/or metabolites) known to influence the variable (data not shown).

**GENDER EFFECTS**

Vitamin B12 status was lower in girls than in boys, as suggested by lower B12 (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 B12 (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. B12 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 B12 in relation to tHcy, MMA, and cystathionine was seen at low B12 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 B12 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 B12, 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 B12 was usually found only in combination with increased methionine.

In logistic regression analyses, low B12 (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 B12 STATUS**

Using ROC analysis, we compared tHcy with other variables related to B12 status (B12, MMA, and cystathionine) in their ability to identify babies with low B12 status. For tHcy, cystathionine, and MMA, it was assumed that a
<table>
<thead>
<tr>
<th>Variable measured</th>
<th>Method (Refs.)</th>
<th>Volume used, µL</th>
<th>Samples selected for analysis</th>
<th>Analyses performed (% of selected)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate</td>
<td>MBA(^b) (25, 27)</td>
<td>4</td>
<td>All samples</td>
<td>4947 (99.2)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>MBA (26, 27)</td>
<td>19</td>
<td>All samples</td>
<td>4874 (97.8)</td>
<td>B(_{12}) &lt;100 pmol/L: n = 159(^c)</td>
</tr>
<tr>
<td>Methionine</td>
<td>MBA (26, 27)</td>
<td>19</td>
<td>All samples</td>
<td>4725 (94.8)</td>
<td>Met &gt;40 µmol/L: n = 154(^c)</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>LC-MS/MS(^d)</td>
<td>15</td>
<td>All samples</td>
<td>4726 (94.8)</td>
<td></td>
</tr>
<tr>
<td>tHcy</td>
<td>EIA (22)</td>
<td>5</td>
<td>All samples</td>
<td>4712 (94.5)</td>
<td></td>
</tr>
<tr>
<td>MMA</td>
<td>GC/MS (21)</td>
<td>15</td>
<td>Random sample</td>
<td>708 (82.8)</td>
<td>n = 993 (19.9%)(^e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tHcy &gt;15 µmol/L</td>
<td>97 (76.4)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B(_{12}) &lt;100 pmol/L</td>
<td>129 (81.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bergen set</td>
<td>92 (88.5)</td>
<td></td>
</tr>
<tr>
<td>MTHFR 677C&gt;T</td>
<td>Real-time PCR  (29)</td>
<td>1</td>
<td>All samples</td>
<td>4962 (99.5)</td>
<td></td>
</tr>
<tr>
<td>Gender (SRY)</td>
<td>Real-time PCR  (28)</td>
<td>1</td>
<td>Random sample</td>
<td>847 (99.1)</td>
<td>n = 1291 (25.9%)(^e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tHcy &gt;15 µmol/L</td>
<td>124 (97.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B(_{12}) &lt;100 pmol/L</td>
<td>158 (99.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bergen set</td>
<td>154 (100.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>101 (96.2)</td>
<td></td>
</tr>
<tr>
<td>CBS mutations</td>
<td>MALDI-TOF MS  (31)</td>
<td>5</td>
<td>Random sample</td>
<td>1133 (98.4)</td>
<td>n = 1327 (26.6%)(^e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>tHcy &gt;15 µmol/L</td>
<td>123 (96.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Met &gt;40 µmol/L</td>
<td>149 (96.8)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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).
baby had low B₁₂ status when the two other metabolites were in the upper quartile and B₁₂ was in the lower quartile. For B₁₂, a low B₁₂ status was defined as tHcy, cystathionine, and MMA being in the upper quartile. “Normal B₁₂ 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 B₁₂ (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 B₁₂.

The relationships between serum B₁₂ and the proportions with increased MMA, cystathionine or tHcy are shown in Fig. 3. The proportion with low B₁₂ 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 B₁₂ concentrations: MMA

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Mean (SD)</th>
<th>g.mean (95% CI)</th>
<th>Percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁₂, pmol/L</td>
<td>4874</td>
<td>355 (203)</td>
<td>395 (300–310)</td>
<td>2.5 5 10 25 50 75 90 95</td>
</tr>
<tr>
<td>Folate, nmol/L</td>
<td>4947</td>
<td>20.3 (12.0)</td>
<td>18.5 (10.8–20.3)</td>
<td>2.5 5 10 25 50 75 90 95</td>
</tr>
<tr>
<td>Methionine, μmol/L</td>
<td>4725</td>
<td>7.5 (4.6)</td>
<td>6.5 (4.3–7.5)</td>
<td>2.5 5 10 25 50 75 90 95</td>
</tr>
<tr>
<td>Cystathionine, μmol/L</td>
<td>6785</td>
<td>0.60 (0.44)</td>
<td>0.52 (0.34–0.60)</td>
<td>2.5 5 10 25 50 75 90 95</td>
</tr>
<tr>
<td>MMA, μmol/L</td>
<td>708</td>
<td>0.31 (0.31)</td>
<td>0.28 (0.25–0.31)</td>
<td>2.5 5 10 25 50 75 90 95</td>
</tr>
</tbody>
</table>

*In the random sample set.*
was within the reference interval, methionine was non-significantly 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 1.7% 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), B₁₂ (P = 0.70), or CMR (P = 0.45). Methionine (g.mean = 20.4, 19.6, and 25.5 μmol/L; P = 0.977) 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, B₁₂, 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 B₁₂ 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 B₁₂ status or possibly another remethylation defect. Thus, our data suggest that measurement of methionine, cystathionine, and B₁₂ 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.

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**Table 3. Spearman correlations between serum variables.**

<table>
<thead>
<tr>
<th></th>
<th>Folate</th>
<th>Methionine</th>
<th>tHcy</th>
<th>Cystathionine</th>
<th>MMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁₂</td>
<td>0.11</td>
<td>-0.03</td>
<td>-0.32</td>
<td>-0.23</td>
<td>-0.28</td>
</tr>
<tr>
<td>Folate</td>
<td>-0.19</td>
<td>0.32</td>
<td>0.06</td>
<td>-0.16</td>
<td>0.00</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.28</td>
<td>0.33</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tHcy</td>
<td>0.36</td>
<td>0.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*If not otherwise stated, the correlation was significant at a level of P < 0.001.

*Correlations including MMA are based on the random sample set, n = 708.

P = 0.030.

P = 0.97.
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.
Fig. 3. Proportions of infants with increased metabolite concentrations according to serum B12 concentrations and with low serum B12 concentrations according to serum tHcy concentrations.

The thresholds to define abnormal concentrations were as follows: 
- MMA: 0.30 μmol/L (73rd percentile); 
- Cystathionine: 0.55 μmol/L (58th percentile); 
- tHcy: 8.0 μmol/L (68th percentile); 
- B12: 220 pmol/L (25th percentile).

* significantly different from those with the highest B12 or lowest tHcy concentrations.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>&lt;5</th>
<th>5–10</th>
<th>10–15</th>
<th>15–20</th>
<th>&gt;20</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>745</td>
<td>3516</td>
<td>597</td>
<td>84</td>
<td>43</td>
</tr>
<tr>
<td>Percentage of population</td>
<td>14.9</td>
<td>70.5</td>
<td>12.0</td>
<td>1.69</td>
<td>0.86</td>
</tr>
<tr>
<td>Vitamin B12 (g.mean), pmol/L</td>
<td>395</td>
<td>304</td>
<td>236</td>
<td>228</td>
<td>258</td>
</tr>
<tr>
<td>Vitamin B12 &lt;123 pmol/L, %</td>
<td>4.5</td>
<td>3.7c</td>
<td>10.9</td>
<td>12.2</td>
<td>8.1c</td>
</tr>
<tr>
<td>Folate (g.mean), nmol/L</td>
<td>18.7</td>
<td>17.6d</td>
<td>16.7</td>
<td>15.1</td>
<td>16.7e</td>
</tr>
<tr>
<td>Folate &lt;7.5 nmol/L, %</td>
<td>4.1</td>
<td>4.7c</td>
<td>6.9c</td>
<td>8.4c</td>
<td>9.8c</td>
</tr>
<tr>
<td>Methionine (g.mean), μmol/L</td>
<td>17.4</td>
<td>20.4</td>
<td>23.7</td>
<td>29.7</td>
<td>35.8</td>
</tr>
<tr>
<td>Methionine &gt;37.3 μmol/L, %</td>
<td>1.0</td>
<td>3.7</td>
<td>11.2</td>
<td>24.1</td>
<td>47.6</td>
</tr>
<tr>
<td>Cystathionine (g.mean), μmol/L</td>
<td>0.40</td>
<td>0.52</td>
<td>0.67</td>
<td>0.69</td>
<td>0.70</td>
</tr>
<tr>
<td>Cystathionine &gt;1.24 μmol/L, %</td>
<td>3.1</td>
<td>4.1</td>
<td>9.1</td>
<td>10.1g</td>
<td>19.1</td>
</tr>
<tr>
<td>MMA (g.mean),e μmol/L</td>
<td>0.21</td>
<td>0.26</td>
<td>0.38</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>MMA &gt;0.59 μmol/L, %</td>
<td>0</td>
<td>4.1f</td>
<td>11.1</td>
<td>20.3</td>
<td>24.2</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Extreme groupa</th>
<th>Methionine, μmol/L</th>
<th>tHcy, μmol/L</th>
<th>Cystathionine, μmol/L</th>
<th>Folate, nmol/L</th>
<th>Vitamin B₁₂, pmol/L</th>
<th>MMA, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (total population)</td>
<td>20.4</td>
<td>7.1</td>
<td>0.51</td>
<td>17.8</td>
<td>302</td>
<td>0.26</td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 11)</td>
<td><strong>69.2 (62.8–115.1)]</strong></td>
<td><strong>10.0</strong></td>
<td><strong>1.02</strong></td>
<td><strong>10.5</strong></td>
<td>251</td>
<td>0.29</td>
</tr>
<tr>
<td>Lowest (n = 10)</td>
<td><strong>1.4 (0.6–2.5)</strong></td>
<td>7.6</td>
<td>0.91</td>
<td>49.0</td>
<td>389</td>
<td>0.26</td>
</tr>
<tr>
<td>tHcy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 10)</td>
<td><strong>41.7</strong></td>
<td><strong>30.2 (28.4–36.8)</strong></td>
<td><strong>0.74</strong></td>
<td>12.9</td>
<td>263</td>
<td>0.31</td>
</tr>
<tr>
<td>Lowest (n = 10)</td>
<td><strong>11.0</strong></td>
<td><strong>2.5 (1.9–2.8)</strong></td>
<td>0.39</td>
<td>21.4</td>
<td>331</td>
<td>0.25</td>
</tr>
<tr>
<td>Cystathionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 10)</td>
<td>22.9</td>
<td>7.6</td>
<td><strong>5.89 (4.04–10.34)</strong></td>
<td>14.5</td>
<td>162</td>
<td>–</td>
</tr>
<tr>
<td>Lowest (n = 10)</td>
<td><strong>7.2</strong></td>
<td><strong>5.4</strong></td>
<td>0.08 (0.02–0.11)</td>
<td>23.4</td>
<td>417</td>
<td>0.22</td>
</tr>
<tr>
<td>Folate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 25)</td>
<td><strong>10.2</strong></td>
<td>6.8</td>
<td>0.55</td>
<td>&gt;80 (&gt;80)</td>
<td>380</td>
<td>0.25</td>
</tr>
<tr>
<td>Lowest (n = 15)</td>
<td>19.5</td>
<td>7.6</td>
<td>0.46</td>
<td>&lt;2.0 (&lt;2.0)</td>
<td>240</td>
<td>0.33</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 162)</td>
<td>19.5</td>
<td>5.6</td>
<td>0.44</td>
<td>20.9</td>
<td>&gt;1000 (&gt;1000)</td>
<td>0.22</td>
</tr>
<tr>
<td>Lowest (n = 79)</td>
<td>19.1</td>
<td>6.2</td>
<td>0.65</td>
<td>14.8</td>
<td>&lt;50 (&lt;50)</td>
<td>0.27</td>
</tr>
<tr>
<td>MMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highest (n = 11)</td>
<td>22.4</td>
<td>11.5</td>
<td>0.83</td>
<td>21.4</td>
<td>170</td>
<td>2.04 (1.35–4.89)</td>
</tr>
<tr>
<td>Lowest (n = 13)</td>
<td><strong>11.2</strong></td>
<td>5.2</td>
<td>0.33</td>
<td>26.9</td>
<td>347</td>
<td><strong>0.11 (0.09–0.12)</strong></td>
</tr>
</tbody>
</table>

- 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.
- 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).
- Results of the extreme group for the selected variable.
- Include fewer than four values, and statistical difference was not tested.
- *P = 0.06.*
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 B12 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). 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 B12 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 B12, 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 B12 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 B12 were consistent with a better B12 status in boys than in girls. There are gender differences in the B12 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 attribu-
utable to low B₁₂ 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 B₁₂ Status**

Our data confirm that many babies have low B₁₂ concentrations (13–15). We found that ~10% had B₁₂ 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 B₁₂ to separate between those with or without biochemical disturbances of B₁₂ status revealed that all four variables had approximately equal discriminatory power.

Published results on B₁₂ 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 B₁₂ 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 B₁₂ and increased tHcy concentrations.

Newborn screening for B₁₂ 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 B₁₂-deficient. More restrictive thresholds combined with a second test could improve diagnostic accuracy and reduce the prevalence. Indeed, diagnosis of B₁₂ deficiency or low B₁₂ status should depend on at least two findings: low B₁₂ combined with clinical symptoms, or low B₁₂ combined with increased metabolites (47). Use of a combination of tHcy >10 μmol/L and B₁₂ <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 B₁₂ 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 B₁₂ deficiency in a baby, which is usually caused by low B₁₂ 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 B₁₂ deficiency in young children (19). Measurement of tHcy, B₁₂, 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 B₁₂ supplements during pregnancy and lactation or to give a B₁₂ supplement to the baby while breastfeeding (19). Yet another possibility is to introduce fortification of B₁₂ 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. B12 deficiency could be one explanation (64), but in samples with extremely low methionine (which consistently had increased folate), B12 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 B6, 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 B6 treatment (71). The possible significance of vitamin B6 status in newborns should be further investigated.

VERY LOW B12 CONCENTRATIONS
A most surprising finding was that babies with the lowest B12 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 B12 function. The explanation for the low B12 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 B12 assay. An alternative explanation is that the active form of B12 in serum, holotranscobalamin, is normal, whereas B12 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 sequelae of B12 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 B12 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 B12 deficiency (e.g., if the mother is a vegetarian) and babies with close family members with homocystinuria.
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.

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