Facts and Recommendations about Total Homocysteine Determinations: An Expert Opinion

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Background: Measurement of plasma total homocysteine has become common as new methods have been introduced. A wide range of disorders are associated with increased concentrations of total homocysteine. The purpose of this review is to provide an international expert opinion on the practical aspects of total homocysteine determinations in clinical practice and in the research setting and on the relevance of total homocysteine measurements as diagnostic or screening tests in several target populations.

Methods: Published data available on Medline were used as the basis for the recommendations. Drafts of the recommendations were critically discussed at meetings over a period of 3 years.

Outcome: This review is divided into two sections: (*a*) determination of homocysteine (methods and their performance, sample collection and handling, biological determinants, reference intervals, within-person variability, and methionine loading test); and (*b*) risk assessment and disease diagnosis (homocystinuria, folate and cobalamin deficiencies, cardiovascular disease, renal failure, psychiatric disorders and cognitive impairment, pregnancy complications and birth defects, and screening of elderly and newborns). Each of these subsections concludes with a separate series of recommendations to assist the clinician and the research scientist in making informed decisions. The review concludes with a list of unresolved questions.

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Increased plasma total homocysteine (tHcy)[7] is a sensitive marker of folate and cobalamin (vitamin B_{12}) deficiency *(1, 2)* and an independent risk factor for cardiovascular disease (CVD) *(3, 4)*. Plasma tHcy concentrations are also related to birth defects *(5)*, pregnancy complications *(6)*, psychiatric disorders *(7)*, and cognitive impairment in the elderly *(8)*. The measurement of tHcy in the clinical setting is thus potentially of great importance *(9)*.

The introduction of tHcy assays in the mid-1980s *(10, 11)* started a new era of research on Hcy. However, it was the advent of immunoassays in the latter half of the 1990s *(12, 13)* that changed tHcy determinations from research tools to widely used clinical chemistry tests. As a result, interest in this field has increased exponentially in both routine diagnostics and research.

Although several reviews on tHcy determinations have been published *(14–18)*, few have provided recommendations for their use in clinical practice *(19–23)*. Our aim was to review the practical aspects of tHcy determinations in clinical practice as well as in the research setting and to survey the data on tHcy in diagnostics or as screening tests in several target populations.

Ideally, guidelines should be established by a multidisciplinary team including all relevant stakeholders, and the recommendations should be according to evidence-based medicine *(24)*. There are not sufficient data in the Hcy field, however, to use such an approach. In particular,

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[7] Nonstandard abbreviations: tHcy, total homocysteine; CVD, cardiovascular disease; MMA, methylmalonic acid; FPIA, fluorescence polarization immunoassay; GC-MS, gas chromatography–mass spectrometry; MS-MS, tandem mass spectrometry; MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine β-synthase; NTD, neural tube defect; and holoTC, holotranscobalamin.

data from controlled clinical trials are sparse. Nevertheless, both clinicians and scientists need guidelines on how to use the tHcy assay and how to interpret the results. Accordingly, we have adopted an approach in which we base our recommendations on an expert opinion, using the evidence available and our long-term experience with tHcy measurements in research and in routine laboratory medicine. Each section provides a summary of the evidence for use of tHcy determinations in a particular setting and concludes with our personal recommendations. Wherever possible, the quality of the evidence available (Table 1) is provided in the associated tables.

Methods

A group of European clinical laboratory and scientific experts was assembled. The group included persons with experience in (*a*) laboratory methods for analysis of tHcy, methylmalonic acid (MMA), and B vitamins; (*b*) large case–control studies and population- and patient-based cohort studies; (*c*) randomized controlled clinical trials of tHcy-lowering therapies; and (*d*) routine clinical chemistry assessment of B-vitamin status and tHcy in patient populations. The literature available on Medline was searched for reports on tHcy and the associated B vitamins up to spring 2003. We also searched for additional relevant publications in books and book chapters. The overall strength of the evidence in these publications was evaluated according to widely used criteria, modified to suit this topic (Table 1). Sections of the review were written by the appropriate experts and then circulated to others in the group. Over a period of 3 years, meetings were held at which the recommendations were discussed until a consensus emerged. Our aim is to present the available facts, highlight areas of ignorance, and make recommendations based on our expert opinion.

Facts and Recommendations in tHcy Determinations and Assessment

The biochemistry of Hcy and its relationship with the B vitamins are shown in Fig. 1 and are discussed in more detail in the relevant sections below. The recommended terminology and abbreviations *(25)* and the basis for tHcy determination are presented in Fig. 2. A summary of the critical factors and criteria for optimum sample handling, assay performance, and data collection is listed in Table 2.

METHODS AND THEIR PERFORMANCE

Methods for tHcy were introduced in the mid-1980s *(10, 11)* and overcame the problems related to the presence of multiple unstable Hcy species in plasma. In all available assays, plasma or serum is initially treated with a reducing agent that converts all Hcy species into the reduced form, HcyH, which is measured either directly or after derivatization (Fig. 2) *(14)*.

For a thorough evaluation of assay design and performance, readers are referred to published reports *(14–18)*. Briefly, the tHcy methods can be classified into two groups: chromatographic methods and enzyme and immunoassays *(16, 17)*. The latter group measure only tHcy

Fig. 1. Hcy metabolism and the enzymes and B vitamins involved.

Hcy is an intermediary in sulfur amino acid metabolism, and it links the methionine cycle (*left*) with the folate cycle (*right*). Hcy is formed from methionine (*Met*) via the numerous adenosylmethionine-dependent methyl transfer reactions (one of the enzymes, guanidinoacetate methyltransferase, catalyzes the formation of creatine and, hence, creatinine). The metabolism of Hcy depends on several B vitamins, including folate, B_{12} (cobalamin), B_6 (pyridoxine), and B_2 (riboflavin). An imbalance between Hcy formation and removal will lead to changes in plasma tHcy concentrations. *Ado*, adenosine; *AdoHcy*, *S*-adenosylhomocysteine; *AdoMet*, *S*-adenosylmethionine; $B12^{+1,+2}$, oxidation state of cobalt atom in B_{12}; *BHMT*, betaine-homocysteine *S*-methyltransferase; CH_2THF, 5,10-methylenetetrahydrofolate; CH_3THF, 5-methyltetrahydrofolate; *CL*, cystathionine γ-lyase; *Cysta*, cystathionine; *DHF*, dihydrofolate; *DHFR*, dihydrofolate reductase; *MAT*, methionine adenosyltransferase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; *MTs*, AdoMet-dependent methyltransferases; *R*, methyl acceptor (e.g., phospholipids, proteins, DNA, RNA, amino acids, and neurotransmitters); *SAHH*, *S*-adenosylhomocysteine hydrolase; *SHMT*, serine hydroxymethyltransferase; *THF*, tetrahydrofolate; *TS*, thymidylate synthase.
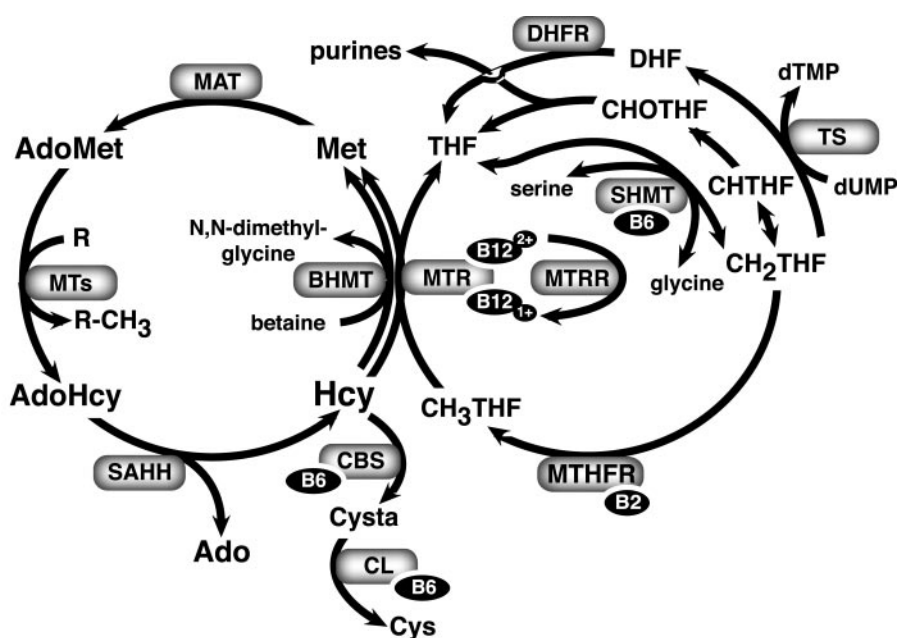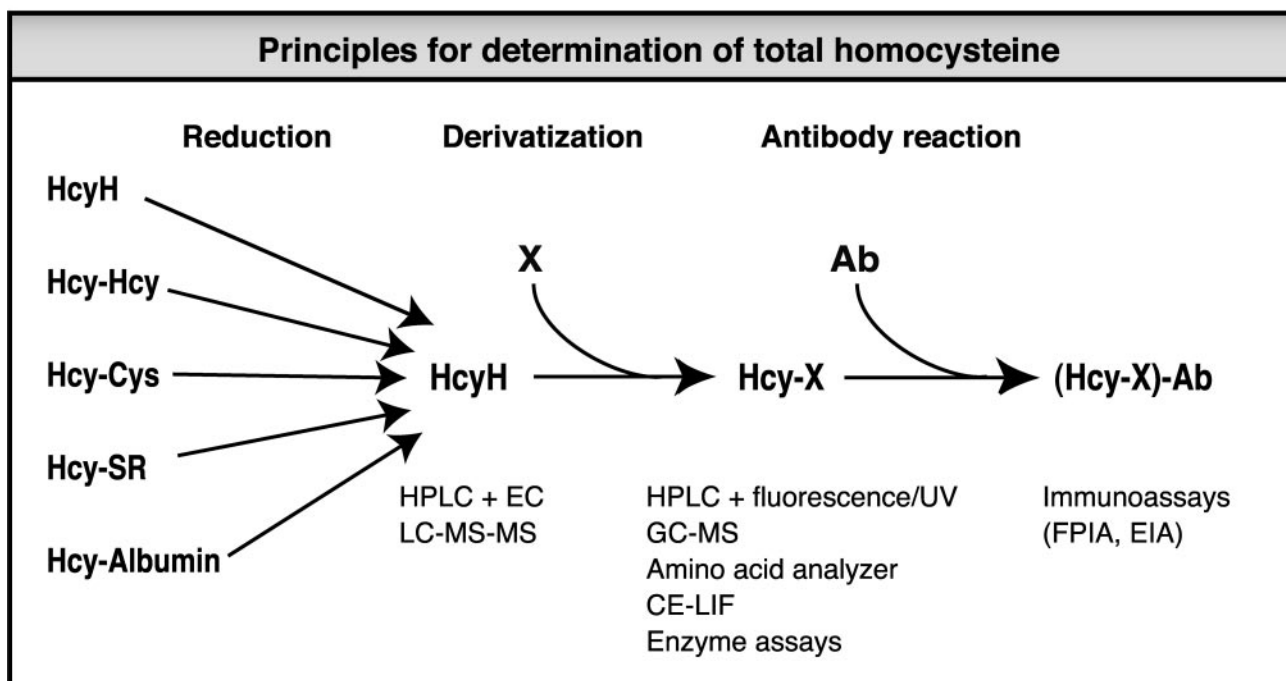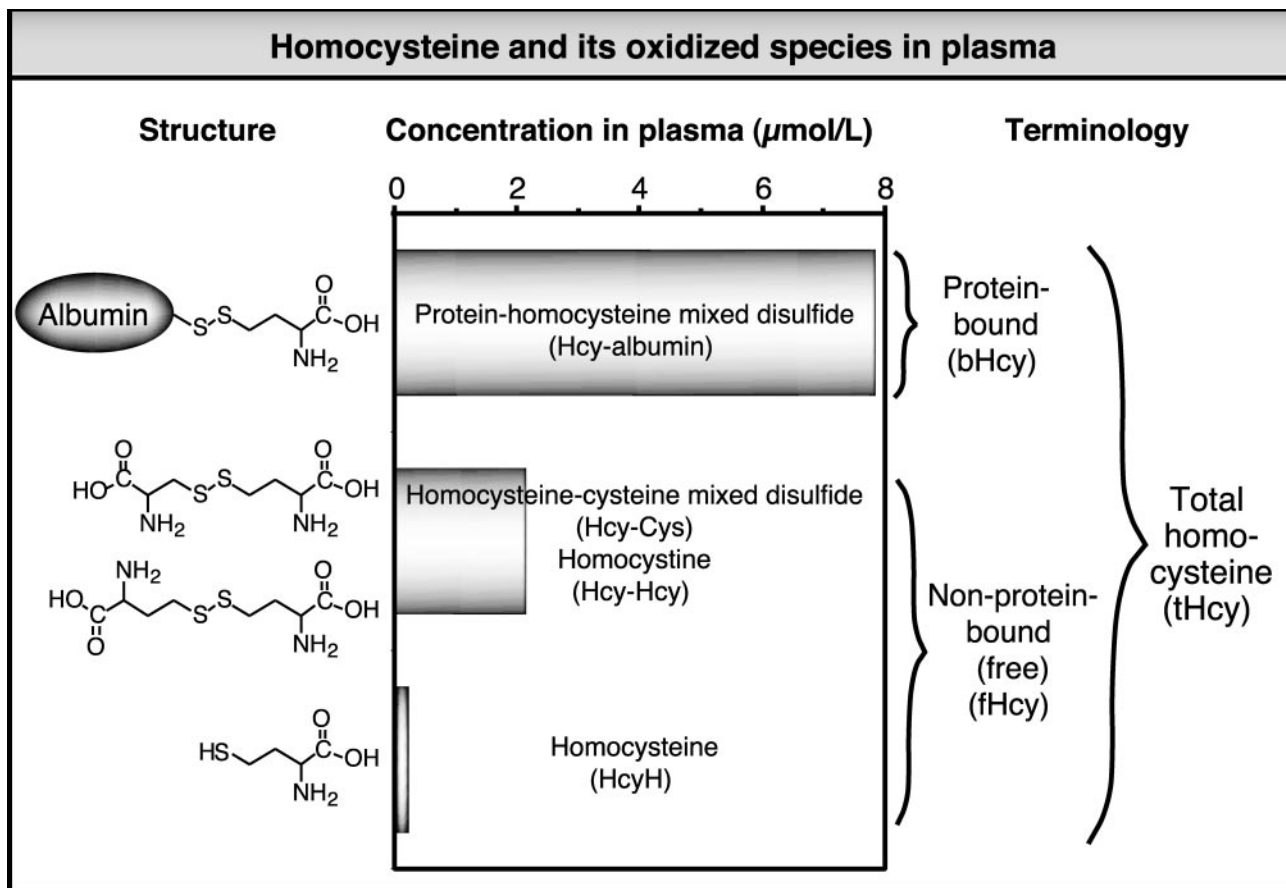
Fig. 2. Hcy and the related disulfides in human plasma, and the principles for determination of plasma tHcy.

(*Top*), Hcy in plasma rapidly becomes oxidized and therefore exists in multiple forms: most exists as a mixed disulfide with albumin, the remainder as free circulating disulfide forms. Only a small proportion remains as the sulfhydryl form, HcyH. Modified from Ueland *(287)* and printed with permission from *Clinical Chemistry*. (*Bottom*), in all tHcy assays, the disulfides, including protein-bound Hcy, are cleaved by treatment with a reducing agent, yielding a single form, *HcyH*, which then is either determined directly or after derivatization. Hence, tHcy is the sum of all Hcy species *(14, 25, 287)*. Ab, antibody; *CE-LIF*, capillary electrophoresis with laser-induced fluorescence detection; *EC*, electrochemical detection; *EIA*, enzyme immunoassay; *Hcy-SR*, Hcy-mixed disulfide; *LC*, liquid chromatography; *UV*, ultraviolet.

Table 2. Critical factors in assessment of tHcy.

| | Routine | | Research[a] | |
Critical or required	Homocystinuria	Other diseases and conditions	Large cohorts/trials with one blood sample	Small sample size, case–control studies, sequential sampling
High accuracy of method	No	Bias <10%	No[b]	No[b]
High precision of method	No	CV <5%	Preferably	CV <5% and/or repeated analyses
Fasting state during blood sampling	No	No	No	Defined
Specific posture during blood sampling	No	No	No	Defined[c]
Defined blood sample handling	No	Yes[d]	Yes[d]	Yes, very critical[c,d]
Data on age, sex, pregnancy	No	Age and pregnancy	Yes[e]	Yes[e]
Data on lifestyle, diseases, and drugs influencing tHcy	No	Preferably	Preferably[e]	Yes[e]
Blood concentrations of vitamins and creatinine	No[f]	No[f]	Preferably[e]	Yes[e]
Methionine loading	No	No	Rarely	Sometimes

[a] Even when not listed as essential, a defined protocol with optimal sample collection and handling, the use of a high-performance method, and extensive data collection could improve the quality of any study. See text for the particular problems concerning within-person variability and regression dilution bias.

[b] If the tHcy results are to be used for establishing reference intervals or will be directly compared with results from other laboratories or studies, the method needs to be accurate.

[c] Avoid systematic differences between cases and controls or between sequential blood samples. This is particularly important when investigating conditions associated with small tHcy differences (<30%).

[d] Centrifugation or cooling of samples should take place within 1 h for routine, 1–2 h for large cohorts or trials, and <30 min or immediate cooling for other studies.

[e] Determinants (e.g., age, sex, smoking, vitamin status, renal function) should be considered during recruitment and the statistical analyses to avoid systematic differences or confounding, and thus false, associations.

[f] Only need to be measured if tHcy is increased.

but are usually simple to perform. The fluorescence polarization immunoassay (FPIA), run on Abbott's IMx and AxSYM platforms (12, 26), is widely used in both research and routine laboratory settings. Chromatographic assays include amino acid analysis; HPLC with ultraviolet, fluorescence, or electrochemical detection; capillary electrophoresis with fluorescence detection; gas chromatography–mass spectrometry (GC-MS); and liquid chromatography with tandem MS (MS-MS) (14, 17, 18). The chromatographic assays usually require skilled staff and are labor-intensive, and throughput may be low (14). The advantages of chromatographic assays include wide analytical range, simultaneous determination of other compounds (e.g., other sulfur amino acids and MMA), and sometimes lower cost than commercial reagent-based assays (14, 17).

The different tHcy methods give comparable results (16, 27), but the variations among methods and among laboratories are considerable (16, 28–31). Ideally, from the known biological inter- and intraindividual variation in tHcy, the bias should be <10% (0.375 × $CV_{between-person}$), and the imprecision no higher than 5% (0.75 × $CV_{within-person}$), but many methods do not fulfill these criteria (29).

There is a need for standardization of tHcy assays (16). Certified reference material is lacking, which is a problem because different types of calibrator materials often yield different values (32). Hcy calibrators in water or plasma often have greater imprecision than plasma-based calibrators (29, 30). The inclusion of an internal calibrator improves performance for some (29, 33) but not all methods (34). The quality of tHcy measurements has improved in

recent years, but the problem of standardization remains unresolved.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- The assay should be cost-effective, fast, robust, easy to perform, and stable over time.
- Inaccuracy (bias) should preferably be <10%, and imprecision should preferably be <5%.
- The analytical range should cover the 0.5th–99.5th percentiles in the general population (~3–40 μmol/L).
- Most newer enzyme and immunoassays fulfill these criteria and are suitable for routine laboratories.
- Because the accuracy of tHcy measurements differs among methods and laboratories, caution should be used in comparing values obtained in different laboratories.
- Participation in an external quality-control program is strongly recommended.

RECOMMENDATIONS IN THE RESEARCH SETTING

- The tHcy method should be appropriate for the study. Analytical range should be considered. Low imprecision is particularly important in studies with small populations and small tHcy differences.
- The ability to simultaneously measure other compounds may be relevant, whereas accuracy, cost, capacity, and practicability may be less critical than in routine laboratories.

- Studies involving several laboratories should use methods with low bias (FPIA, some GC-MS assays) and establish joint procedures for calibration.
- Chromatographic assays may be best suited for laboratories with experience in such technology, whereas the enzyme and immunoassays are simple to set up and thus are suitable for any laboratory.
- Participation in an external quality-control program is strongly recommended.

SAMPLE COLLECTION AND HANDLING

Food intake and diurnal and seasonal variations. A small meal will not influence tHcy concentrations in healthy people (35, 36), whereas intake of a large, protein-rich meal may increase the plasma tHcy concentration by ~10–15% after 6–8 h (36, 37). This may explain the diurnal variation, with tHcy concentrations being lowest in the first part of the day and highest in the evening (38). Plasma tHcy is probably not subject to seasonal variation (39, 40).

Posture during blood collection/venous stasis. The tHcy concentration is not altered by the duration of venous stasis (15). Blood samples collected in the supine position have ~10% lower mean tHcy concentrations than those collected in the sitting position (15), possibly because plasma albumin (which binds Hcy) is reduced in the supine position. This phenomenon may contribute to the lower tHcy concentrations observed in the acute phase after myocardial infarction compared with samples collected 2–3 months later (41, 42). Likewise, comparison of tHcy concentrations in patients confined to bed with those in healthy (sitting) controls may bias results of case–control studies.

Plasma vs serum. Traditionally, it has been recommended that tHcy should be measured in plasma because the use of anticoagulant allows immediate sample processing. Serum, even if optimally prepared, yields slightly higher values than plasma (see below). Optimally collected EDTA or heparin plasma gives identical results, whereas citrated plasma yields 5–15% lower tHcy values (43). The influence of coagulant also depends on the method used. For example, EDTA improves the fluorescence yield in the monobromobimane assay (32) but is not compatible with some of the GC-MS methods (11).

tHcy export from blood cells and use of stabilizers. After blood collection, but before removal of the blood cells, there is a time- and temperature-dependent increase in tHcy (Fig. 3). This is attributable to an ongoing release of Hcy from erythrocytes (44). At room temperature, the increase in tHcy is ~1 μmol $\cdot$ L^{-1} $\cdot$ h^{-1}, but it is not dependent on the initial tHcy concentration (43). Hence, this corresponds to an ~10% increase per hour in a typical sample with 10 μmol/L tHcy, but only 3% in a 30 μmol/L sample (43). Because serum is prepared from a blood sample left at room temper-
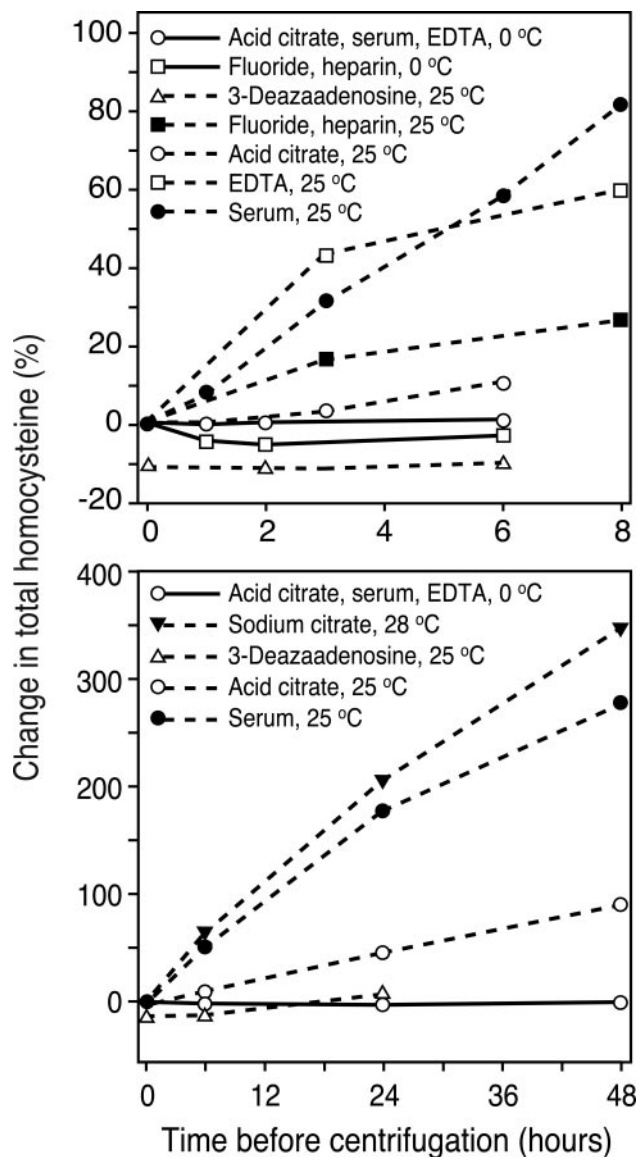

Fig. 3. Time- and temperature-dependent increase in tHcy in the plasma/serum fraction of whole blood supplemented with various "stabilizers" or anticoagulants.

Plasma or serum tHcy was determined by FPIA. Data were kindly provided by C. Westby, Axis-Shield ASA, Oslo, Norway (45).

ature for 30–60 min to allow coagulation, serum concentrations will usually be ~5–10% (~0.5–1 μmol/L) higher than those obtained in optimally prepared plasma (15).

The increase in tHcy is prevented by immediate centrifugation and removal of the blood cells or by keeping samples cooled on ice until centrifugation (15). The use of gel separator tubes that are rapidly centrifuged also prevents the increase of tHcy in serum for at least 48 h (45).

Several stabilizers may prevent the formation or release of Hcy from the blood cells (15, 46). Sodium fluoride appears to reduce the increase, but this is probably attributable to an osmotic effect on the red cells, diluting the plasma (46). Adenosine analogs, such as 3-deazaadenosine, are effective

but not compatible with assays based on *S*-adenosylhomocysteine hydrolase, including the FPIA *(47)*. Acidic citrate stabilizes the tHcy concentrations for a few hours, but its use usually leads to small but consistent changes in tHcy *(46)*. Determination of tHcy in whole-blood lysates has been suggested as an alternative to plasma *(48)*, but this will require a new set of reference intervals.

Overall, the problem with stabilization of tHcy in whole blood is only partly solved, and use of stabilizers usually leads to small but systematic deviations at baseline *(46)*. Most experts therefore still recommend that plasma is prepared optimally with cooling or immediate centrifugation.

Stability of tHcy in stored plasma/serum. After removal of the blood cells, tHcy in plasma or serum is stable. No changes are observed for at least 4 days at room temperature *(43)*, for several weeks in the refrigerator, or for several years at −20 °C *(15)*. Freeze–thaw cycles are usually tolerated well *(15)*; however, after freezing, our experience is that inhomogeneity of the sample matrix is a common problem. Hence, thorough mixing of the samples is required after thawing.

Hemolysis. Hemolysis may interfere with some tHcy assays but will usually not change the plasma tHcy concentrations per se. Although the tHcy concentration in erythrocytes is lower than in plasma *(48)*, hemolysis has to be extensive to change the tHcy concentration even by a small percentage *(43)*.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- The laboratory should provide instructions about procedures for sample collection and handling. For commercial assays, the instructions provided by the manufacturers should be followed.
- Strict directions on time since last meal, posture during blood sampling, and duration of venous stasis are not necessary because these factors are unlikely to affect the interpretation of the results.
- One type of collection tube should be recommended. EDTA tubes are most widely used, but use of serum or citrated or heparinized rather than EDTA plasma will not materially influence the results.
- Blood samples should be centrifuged within 1 h or kept cold until centrifugation (<8 h).
- Plasma/serum samples can be mailed to the laboratory at room temperature. Centrifuged gel separator tubes can also be used.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Given the problems with changes in tHcy after blood collection, a detailed protocol for sample collection, processing, and storage should be prepared for each project.
- Centrifugation and removal of the blood cells, or cooling of the sample, should take place as soon as possible. Uncentrifuged blood that has been kept at room temperature for >2 h is of little value.
- In case–control studies or studies with repeated sampling, protocols should be strictly standardized. Time of day, fasting state, and posture during sample collection should be uniform because minor but systematic alterations in procedures may influence the results and, thus, the outcome of the study.
- In longitudinal studies, samples from the same individual should be analyzed in the same run, whereas in case–control studies, each run should include cases and controls in random sequence. Samples from matched cases and controls should be analyzed together in the same run.
- In large cohort studies, the need for standardization is less strict because variations in sample handling and analysis probably occur randomly and will, if anything, tend to attenuate a finding.

BIOLOGICAL DETERMINANTS

Determinants of plasma tHcy include genetic, physiologic, and lifestyle factors; various diseases (Table 3); and drugs (Table 4). Many of these factors cause a change in tHcy concentrations by altering the function or blood concentrations of the B vitamins, in particular folate and cobalamin, and/or by influencing renal function or, more rarely, by influencing enzyme activities. The causes of increased tHcy concentrations vary according to the age of the person and the degree of tHcy increase (Table 5).

Low folate or cobalamin status or renal impairment account for the majority of cases with increased tHcy *(49–53)*. In populations eating food fortified with folic acid, renal impairment and cobalamin deficiency are the most important determinants *(54)*. Homozygosity for the methylenetetrahydrofolate reductase (MTHFR) 677C→T polymorphism is the most common genetic determinant *(55, 56)*. Individuals with the MTHFR 677TT genotype usually have ~2.5 μmol/L higher tHcy than those with the 677CC variant *(57, 58)*, but it depends on the folate *(59, 60)* and riboflavin status *(61, 62)*. Most other genetic polymorphisms in enzymes related to Hcy have little effect on tHcy concentrations *(63)*.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- In individuals with hyperhomocysteinemia, all of the factors mentioned above should be considered as possible targets for examination, for intervention, or for clinical advice.
- Factors that influence tHcy should be taken into account when interpreting tHcy results.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Studies on tHcy should, if possible, consider the common and important genetic (MTHFR 6777C→T polymorphism), physiologic (age, sex, pregnancy, menopausal state, renal function), lifestyle (smoking, coffee intake, diet, drugs), and blood (folate, cobalamin, creatinine) determinants of tHcy.

REFERENCE INTERVALS

In general, the reference intervals are calculated as the 2.5th–97.5th percentile interval (or 95% reference interval) for presumed healthy individuals. The blood samples should be collected and handled under conditions reflecting usual clinical practice. Reference intervals may be established for different populations to account for important differences, including those related to nonmodifiable factors such as age, gender, or ethnicity, as well as modifiable factors, such as nutritional status, lifestyle, and disease. Some of these will be briefly mentioned below.

Age, gender, and pregnancy. tHcy concentrations increase throughout life (Fig. 4) and approximately double from childhood to old age. After puberty, males have higher mean tHcy concentrations than females. The gender difference in mean tHcy is ~2 μmol/L, but it becomes less with increasing age *(64, 65)*, and the proportion with tHcy above a given upper reference limit is similar in adult men and women. During pregnancy, both the mean concentrations and upper reference limits for tHcy are markedly lower, a finding that is only partly explained by the hemodilution and reduced plasma albumin during pregnancy *(66, 67)*.

Renal function and creatinine. tHcy is dependent on renal function and creatinine synthesis *(68–71)*. Usually, the reference limits for tHcy are calculated after excluding persons with increased creatinine or impaired renal function. Another possibility is to establish different reference limits for different creatinine concentrations, for example, by use of a nomogram. Such data are currently not available.

Nutritional status and lifestyle. The marked effect of vitamin status on the reference intervals highlights the problem of defining "presumed healthy individuals". In most adults who do not eat food fortified with folic acid, the upper reference limit is 15–20 μmol/L or even higher *(64, 72, 73)*. However, in adults with good vitamin status

Table 3. Determinants of plasma tHcy (for drugs, see Table 4).

Causes/determinants	Effect[a]	Evidence[b]	Refs.
Genetic factors			
Homocystinuria[c]	↑↑↑	II/III	*(70)*
Heterozygosity for CBS defects[d]	↑	III	*(97, 104)*
Down syndrome	↓	III	*(238)*
MTHFR 677C→T (homozygosity)	↑	II	*(58, 60)*
Other polymorphisms	(↑)↓	III	*(63)*
Physiologic determinants			
Increasing age	(↑)	II	*(64, 94)*
Male sex	(↑)	II	*(94, 239)*
Pregnancy	↓	II	*(66, 197)*
Postmenopausal state	(↑)	II	*(94, 240)*
Renal function, reduced GFR[e]	(↑)	II	*(68, 69)*
Increasing muscle mass	(↑)	III	*(241)*
Lifestyle determinants			
Vitamin intake (folate, B_{12}, B_6, B_2)	↓	I/II	*(50, 52, 61, 73)*
Smoking	(↑)	II	*(52, 239)*
Coffee	(↑)	I	*(242, 243)*
Ethanol intake	↑↓	II	*(244–246)*
Exercise	(↑)↓	II	*(239, 247)*
Clinical conditions			
Folate deficiency	↑↑	I/II	*(2, 116, 117)*
Cobalamin deficiency	↑↑↑	II	*(2, 117, 234)*
Vitamin B_6 deficiency[d]	↑	III	*(95)*
Renal failure	↑↑	II	*(68, 248)*
Hyperproliferative disorders	↑	III	*(249, 250)*
Hypothyroidism	↑	III	*(251, 252)*
Hyperthyroidism	↓	III	*(252, 253)*
Early stage of diabetes	↓	II	*(69, 254)*
Late stage of diabetes	↑	II	*(254, 255)*

[a] ↓ = decrease in tHcy; (↑) = increase within the reference interval; ↑, ↑↑, and ↑↑↑ = moderate (15–30 μmol/L), intermediate (30–100 μmol/L), and severe hyperhomocysteinemia (>100 μmol/L), respectively.

[b] See Table 1 for definitions.

[c] Homocystinuria includes deficiency in CBS, MTHFR, methionine synthase, or methionine synthase reductase and defects in intracellular cobalamin metabolism (see Fig. 1).

[d] In individuals with vitamin B_6 deficiency or mild defects in CBS, the fasting concentrations are usually within the reference interval, whereas the post-methionine-load tHcy concentrations are often increased.

[e] GFR, glomerular filtration rate.

or a healthy lifestyle, the upper reference limit is ~12 μmol/L (Fig. 5) *(72, 74, 75)*. The introduction of folic acid fortification in the US has markedly reduced the prevalence of hyperhomocysteinemia *(76)*. In adults eating a typical but nonfortified diet, the use of folic acid supplements or a healthy lifestyle will lower the mean tHcy concentrations by 10–30%, and the upper reference limits will be reduced by a similar proportion *(72, 77, 78)*.

Ethnicity. Plasma tHcy concentrations differ among ethnic groups *(64, 79)*, but the effect on the upper reference limit is relatively small between groups living in the same area and eating a similar diet *(64)*. In some regions of the world, in particular in developing countries, tHcy concen-

Table 4. Drug effects on plasma tHcy.[a]

Class	Drug	tHcy	Possible mechanism	Refs.
Folate antagonists	Methotrexate	↑	Inhibition of DHFR[b]	(249, 250)
	Trimethoprim	↑	Inhibition of DHFR	(256)
	Anticonvulsants (inducers)	↑	Inhibition of polyglutamation, folate depletion	(257)
	Cholestyramine	(↑)	Inhibition of folate absorption	(258)
Cobalamin antagonists	Nitrous oxide	↑ ↑	Inactivation of methionine synthase	(259)
	Nitric oxide	ND	Inactivation of methionine synthase	(260)
	Metformin	(↑)	Inhibition of cobalamin absorption	(261)
	H2-receptor antagonists	ND	Inhibition of cobalamin absorption	(262)
	Omeprazole	ND	Inhibition of cobalamin absorption	(262)
Vitamin B₆ antagonists[c]	Niacin	↑	Inhibition of pyridoxal kinase	(263)
	Azauridine	↑	Inhibition of pyridoxal kinase	(264)
	Isoniazid	ND	Inhibition of pyridoxal kinase	(262)
	Theophylline	↑	Inhibition of pyridoxal kinase	(95)
Hcy production	Adenosine analogs	↓	Inhibition of AdoHcy hydrolase	(265)
	Creatine	↓[d]	Reduced creatinine (and Hcy) synthesis	(266, 267)
	L-Dopa	↑	Substrate for AdoMet-dependent COMT	(268, 269)
Sulfhydryl compounds	D-Penicillamine	↓	Disulfide exchange, displacement	(270)
	N-Acetylcysteine	↓	Disulfide exchange, displacement	(271, 272)
	Mesna	↓	Disulfide exchange, displacement	(273)
Sex steroids and related compounds	Estrogens (postmenopausal)	↓	Not known, interference with vitamin function	(240)
	Androgens	(↑)	Increased muscle mass/creatinine synthesis	(274)
	Tamoxifen	↓	Not known, estrogen effect?	(275)
	Aminoglutethimide	↑	Induction of liver metabolism	(276)
Other	Betaine	↓	Enhancement of remethylation	(277)
	Cyclosporin A	↑	Impaired renal function	(278, 279)
	Simvastatin	↓	Not known	(280)
	Fibrates	↑	Renal impairment, altered creatinine metabolism	(281, 282)
	Diuretics	↑	Reduced glomerular filtration rate?	(96, 283)

[a] The data are based on systematic review of the literature [modified from Ref. (262)]. With few exceptions, the level of evidence for each drug is III (Table 1), but the known effects of these drugs support the findings.
[b] DHFR, dihydrofolate reductase; ND, not determined; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; COMT, catecholamine-O-methyltransferase.
[c] Vitamin B₆ antagonists predominantly affect post-methionine-load tHcy concentrations.
[d] Marginal or absent effect.

trations may be very high in the general population. For example, in a group of presumed healthy Asian Indians, the 95th percentile was >50 μmol/L, a finding that was only partly explained by their low cobalamin status (80).

Evaluation. There are two opposing needs: on the one hand, simplicity with few thresholds; on the other hand, the ability to identify those individuals with a truly abnormal value, taking all relevant factors into account. Age, pregnancy, and renal function are important (Table 6). The intake of folic acid as either supplements or through fortification of foods must also be considered (Table 6).

The statistically defined reference interval as discussed here may be different from the desirable tHcy concentrations (81). Because nearly everyone can lower their tHcy by increasing their vitamin intake, it has been suggested that the tHcy reference limits should be based on individuals who are vitamin replete (74, 75). However, it is still not known whether such low tHcy concentrations will have any beneficial effect on health. Hence, until more evidence is available, the conven-tional approach to distinguishing abnormal from normal, i.e., the statistical upper reference limit, should be used.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Each laboratory should establish reference limits for its region.
- The reference population should exclude individuals with folate or cobalamin deficiency or increased creatinine as well as individuals with diseases or who are taking drugs that increase tHcy concentrations.
- Separate reference limits for children, adults, the elderly, and pregnant women should be used (Table 6).
- In a population taking folic acid supplements or eating a folic acid-fortified diet, the upper reference limit is usually 20–25% lower than in the nonfortified population (Table 6).

Table 5. Causes of increased tHcy according to age and the tHcy concentration.

Category	Common causes[a]	Level of evidence[b]
According to age		
Newborns	Cobalamin deficiency	II
	Hypermethioninemia[c]	III
	(Homocystinuria)[d]	III/IV
Children	Folate or cobalamin deficiency[d]	III/IV
	(Homocystinuria)[d]	III/IV
Adults	Unhealthy lifestyle and poor diet (low folate)	I/II
	Vegetarian diet (low cobalamin)	II
	MTHFR 677C→T polymorphism	II
Elderly	Cobalamin deficiency (malabsorption)	II
	Unhealthy lifestyle and poor diet (low folate)	II
	Renal impairment	II
	Drugs	III
According to tHcy concentrations (prevalence in population)[e]		
15–30 μmol/L (<10%)	Unhealthy lifestyle, poor diet	I/II
	Vegetarian diet	III
	MTHFR 677C→T polymorphism	II
	Mild folate or cobalamin deficiency	I/II
	Impaired renal function	II
	Drugs affecting tHcy, folate, or cobalamin	III
	Suboptimal blood sample handling	
30–100 μmol/L (<1%)	Moderate/severe cobalamin deficiency	II
	Moderate/severe folate deficiency	II
	Renal failure	II
>100 μmol/L (<0.02%)	Severe cobalamin deficiency	III/IV
	Homocystinuria	III/IV

[a] In North America, after introduction of folic acid fortification, folate status and the MTHFR 677C→T polymorphisms are no longer important causes of increased tHcy *(76)*.

[b] See Table 1 for definitions.

[c] Babies with high methionine usually have high tHcy and cystathionine, probably explained by nutrient intake or, rarely, by enzymatic defects *(70)*.

[d] Homocystinuria or vitamin deficiency is not common, but testing of a child is usually performed only when there are definite symptoms or suspicion of disease.

[e] Prevalence data based on adults 40–67 years of age, not receiving folic acid fortification *(56, 239)*.

- The upper tHcy reference limit should not be used strictly but interpreted with age, sex, and determinants in mind. In a nonfortified group, a tHcy concentration of 14 μmol/L is high in a young nonsmoking woman. In a 90-year-old man, a concentration of 20 μmol/L is common and does not necessarily indicate disease.

RECOMMENDATIONS IN THE RESEARCH SETTING

- In case–control studies, the controls should optimally be recruited from the same general population as the cases. The cases and controls should have a similar age and gender distributions. Matching and/or controlling for important lifestyle factors should always be considered.

WITHIN-PERSON VARIABILITY

The within-person variability refers to the relationship between repeated testing on the same person at different time points. In the clinical routine, the within-person variability reflects the reliability of a single measurement, and it determines the magnitude of change between two measurements that is significant, for example, in response to treatment or disease. In research studies, the within-person variability has important implications for the power of the study.

The total within-person variability is the sum of within-person biological and analytical variability and is usually given as the within-person SD or CV. The within-person CV for tHcy is ~8% in healthy adults over a 1-year period *(29)*. Hence, in an individual with a true mean tHcy of 10 μmol/L, retesting would yield a value between 9.2 and 10.8 μmol/L in ~70% and 8.4 and 11.6 μmol/L in 95% of the tests. The within-person variability for tHcy may seem substantial but is comparable to the variability for total cholesterol and systolic blood pressure *(40)*.

Hyperhomocysteinemia is associated with higher within-person variability. In individuals with tHcy >40 μmol/L *(56)*, the within-person variability is ~25% after
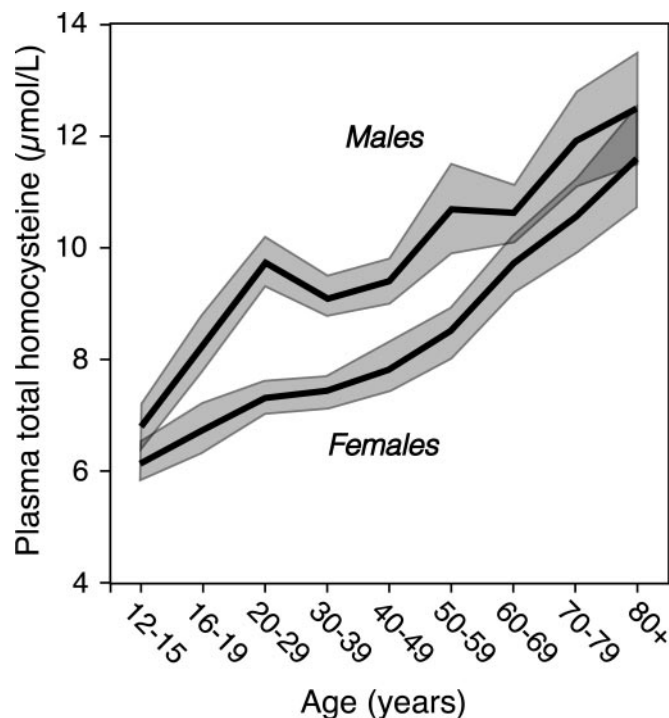
Fig. 4. Effect of age and gender on tHcy concentrations in adolescents and adults.

The *graph* is based on results from the Third National Health and Nutrition Examination Survey carried out before folic acid fortification started in the US. This cohort includes a total of 8585 individuals >12 years from three different groups: non-Hispanic whites, non-Hispanic blacks, and Mexican Americans. Results from the combined race–ethnicity group are presented. Data are given as geometric means, and the *shaded areas* represent 95% confidence intervals. Data were kindly provided by Dr. P. Jacques, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA *(64)*.

Fig. 5. Effect of lifestyle on the tHcy distribution in men (*bottom*) and women (*top*) 40–42 years of age.

Data are presented as distribution curves and as box plots [median (*lines inside boxes*), 25th–75th percentiles (*limits of boxes*), and 2.5th–97.5th percentiles (*horizontal lines*)]. The *dashed lines* represent individuals with a healthy lifestyle (nonsmokers with high folate intake and drinking <1 cup of coffee/day), whereas the *solid lines* represent individuals with an unhealthy lifestyle (smokers drinking ≥5 cups of coffee/day and with low folate intake). Adapted from Nygård et al., 1998 *(72)* with permission of the *American Journal of Clinical Nutrition* © Am. J. Clin. Nutr.; American Society for Clinical Nutrition.

4–8 months and ~35% after ~2 years. A similarly high variability has been observed for persons with cobalamin deficiency *(82)*. Changes in factors known to influence tHcy (Tables 3 and 4) are likely to increase variability.

To decide whether there is a significant change in the tHcy concentration between two measurements from the same individual, both the analytical CV and the biological within-person variability must be considered. This so-called critical difference [$2.77 \times (CV_{analytical}^2 + CV_{biological}^2)^{1/2}$] will be at least 20% *(83)* or, for most laboratories, substantially higher. In relation to tHcy measurements, the size of the analytical CV relative to the biological within-person CV is quite large (~50%). Hence, the magnitude of the critical difference can be reduced by simply testing more replicates of each sample *(84)*.

In cross-sectional or retrospective studies, a single measurement of tHcy in each individual will typically underestimate the true strength of association of tHcy with disease by ~15% *(40)*. In prospective studies, a single tHcy measurement at enrollment may not reflect the individual's long-term mean tHcy concentration, a phenomenon referred to as the regression dilution bias. The magnitude of this bias is greater in studies with longer follow-up intervals. Failure to correct for regres-
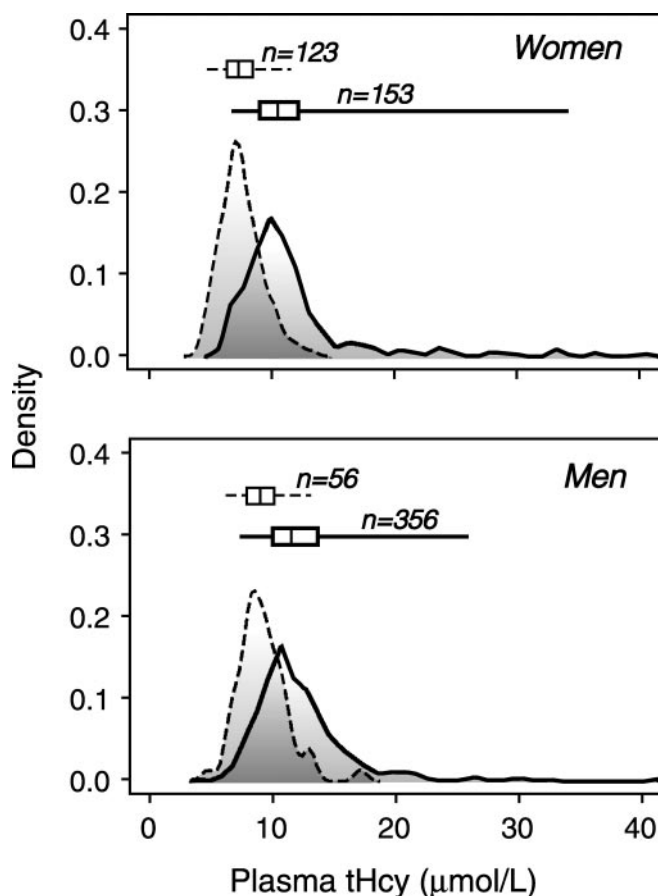
sion dilution may underestimate the relative risks of disease by ~20% after 2 years and 50% after 10 years *(85)*.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- A single tHcy measurement usually reflects the mean tHcy concentration and is adequate in most settings.
- A tHcy change >25–30% between samples collected on two occasions is likely to be significant.
- Repeat measurements taken 1–3 years apart are unlikely to change by more than 20–25% in the absence of onset of disease, medical treatment, or change of lifestyle. Thus, when tHcy measurements are used as a screening variable, 3–5 years between testing seems a reasonable interval.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Replicate measurement of the same sample will reduce the within-person variability by reducing analytical variability.
- In healthy people, measurement of tHcy in a single sample is sufficient for most research purposes.
- In patient populations, the variability is probably greater. Therefore, measurements from two blood samples collected 2–4 weeks apart will improve the quality of such studies.
- Prospective studies with long follow-up time should include repeat measurements some years after the initial measurement in all or in a random sample to correct for the regression dilution bias.

THE METHIONINE LOADING TEST

The methionine loading test was originally developed to diagnose heterozygosity for cystathionine β-synthase (CBS) deficiency (86). Currently, the test is used to identify individuals with mild impairment of Hcy metabolism, in particular in CVD patients, in whom fasting tHcy may be normal but the postload tHcy concentration is increased (87, 88).

Methionine loading involves intake of 100 mg methionine/kg of body weight and measurement of tHcy usually 4–6 h after methionine ingestion (3). A 2-h postmethionine tHcy has been validated and may be more practical in the clinical setting (89), but it probably has greater within-person variability than the 4- or 6-h test (90). There are few side effects of methionine loading (91),

but one possible death from severe overdose was reported recently (92).

In adults, the mean post-methionine-load tHcy measured at 4 or 6 h is ~30 μmol/L, i.e., a 20 μmol/L increase above the fasting value, or 3 times the fasting value (3, 83, 93). The upper reference limit is ~5 times the fasting tHcy concentration (Table 6). Several factors are associated with increased post-methionine-load tHcy concentrations, including higher age; male sex; impaired renal function; low concentrations of folate, cobalamin, and vitamin B_6; the MTHFR 677TT genotype; and heterozygosity for CBS deficiency (90, 94–98). However, with the exception of impaired B_6 function and heterozygosity for CBS deficiency (95, 97), most of these factors increase both the pre- and postload tHcy concentrations. For example, in the European COMAC cohort, (3, 88), among the controls with postload tHcy >50 μmol/L (~95th percentile), only 5% had a fasting tHcy <10 μmol/L (approximately the median). Hence, the majority of individuals with increased post-methionine-load tHcy have fasting tHcy concentrations either above normal or in the high normal range.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- The methionine loading test is cumbersome, and its clinical value is uncertain. The test should be reserved for research purposes.

RECOMMENDATIONS IN THE RESEARCH SETTING

- The methionine loading test could be considered in research on CVD and in studies on regulation of Hcy and its possible physiologic effects.

Facts and Recommendations for the Use of tHcy in Diagnosis and Risk Assessment

There are three main indications for determining tHcy (Table 7): (a) to diagnose homocystinuria; (b) to identify individuals with or at risk of developing cobalamin or folate deficiency; and (c) to assess tHcy as a risk factor for CVD and other disorders. The most important of these will be covered below. Data on tHcy measurement exist for many conditions not discussed here (e.g., vitamin B_2 and B_6 status, cancer, psoriasis, rheumatoid arthritis, and fibromyalgia), but the evidence is insufficient to recommend the use of tHcy on a routine basis in these situations.

Each section below will have the following plan. The facts will first be reviewed under the headings "target populations", "background for using tHcy", and "use of tHcy in risk assessment/diagnosis". We will then give recommendations separately for the routine clinical and research setting. For all sections, the relevant information

Table 6. Upper reference limits for tHcy (μmol/L) in various groups.[a]

Group	Folate supplemented[b]	Nonsupplemented
Fasting/basal tHcy, μmol/L		
Pregnancy	8	10
Children <15 years	8	10
Adults 15–65 years	12	15
Elderly >65 years	16	20
Post-methionine-load tHcy (4–6 h)[c,d]	5 times fasting tHcy, or 40 μmol/L increase above fasting tHcy (adults)	

[a] Data are based on our assessment of published literature, including a more detailed analysis of data from large populations studies such as the Third NHANES (64), the Hordaland Homocysteine Study (72), The Jerusalem Lipid Research Clinic Study (284), The Oxford Healthy Aging Project (OHAP) (53), British National Diet and Nutrition Survey (285), The European Concerted Action Project (88), and the children study in Oslo (286).

[b] Individuals eating folic acid-fortified food or taking folic acid-containing supplements.

[c] Results from 800 adults 20–60 years of age in a European population (no folic acid fortification) (3, 88). Similar results were obtained by Fokkema et al. (83).

[d] Post-methionine-load tHcy after the 2-h test is ~75% of the 4-h test (89).

Table 7. Target populations for use of tHcy measurements.

Target group	Reason for measuring tHcy	When and how often to test[a]	Level of evidence[b]
Patients with symptoms of or at risk of folate or cobalamin deficiency	To exclude or confirm folate and/or cobalamin deficiency	See Fig. 6	II, III
Patients treated for folate or cobalamin deficiency	To monitor response to vitamin treatment, or to detect relapse	2–4 weeks after start of therapy, then once a year, or if symptoms arise	I, II
Patients with symptoms of homocystinuria or siblings of homocystinuric patients	To exclude or confirm homocystinuria	At entry into the medical system (once)	III, IV
Patients with homocystinuria	To monitor treatment response and compliance	Every 2–4 weeks until tHcy is stable, then once a year, or after change in treatment regimen	III, IV
CVD patients or patients at high CVD risk	To identify those at high risk of CVD events and mortality and to exclude homocystinuria	At entry into the medical system; possibly every 3–5 years[c]	II

[a] These recommendations are based on our opinions.
[b] See Table 1 for definitions.
[c] Increased tHcy should be used as a prognostic factor; it is therefore reasonable to measure it on a regular basis.

related to tHcy determinations and assessment in both the clinical and the research setting is summarized in Table 2.

HOMOCYSTINURIA

Target populations. The target populations include patients, in particular children and young adults, with symptoms of homocystinuria, including thromboembolism, lens dislocation, progressive myopia, osteoporosis, marfan-like appearance, unexplained mental retardation, psychiatric disorders, or megaloblastic anemia, as well as siblings or children of patients with homocystinuria.

Background. Homocystinuria refers to the rare inborn errors of metabolism leading to urinary excretion of large amounts of homocystine combined with severely increased plasma tHcy concentrations, usually >100 μmol/L (25). The most common cause is CBS deficiency, but impaired Hcy remethylation attributable to defects in methionine synthase, in MTHFR, or in factors or enzymes involved in the transport or metabolism of cobalamin may also occur (99). Independent of site of defect, these patients have a high risk of premature, frequently fatal, thromboembolic events (70).

CBS deficiency is an autosomal recessive disease. The reported worldwide birth prevalence is ~1 in 300 000, but the prevalence is higher in Ireland and New South Wales (70, 100). Data from Scandinavia based on genetic analyses in newborns (101) and extensive tHcy testing of the population show that CBS deficiency may be much more common, i.e., at least 1 in 20 000 live-born children.

CBS deficiency is divided into pyridoxine-responsive and -nonresponsive variants, depending on the effect of pyridoxine on the tHcy concentration (70). Patients with pyridoxine-nonresponsive CBS deficiency often have a severe clinical phenotype with early symptom debut, but both the responsive and nonresponsive groups may suffer from serious complications (70). Lens dislocation and myopia are usually the earliest signs, but some patients experience few typical symptoms and are first diagnosed

after a CVD event (102). Early diagnosis and treatment with pyridoxine and/or folic acid and betaine, preferably from infancy, can prevent CVD events and most of the clinical symptoms (100, 103). The beneficial effect of tHcy-reducing therapy is independent of pyridoxine responsiveness (100).

Most individuals heterozygous for CBS deficiency have normal fasting tHcy, but their urinary tHcy concentrations may be increased (97), and some, but not all, respond to a methionine loading with an abnormal increase in tHcy (97, 104). Increased postload tHcy concentrations are a risk factor for vascular disease (88) and neural tube defects (NTDs) (105), but heterozygosity for CBS deficiency is not a frequent finding in these conditions (106, 107), and heterozygous individuals do not seem to be at increased CVD risk (70).

Compared with CBS deficiency, less is known about the inborn errors leading to impaired Hcy remethylation. These defects usually, but not always, become clinically apparent early in life and are associated with developmental delay, failure to thrive, megaloblastic anemia, and myelopathy (99). In MTHFR deficiency, megaloblastic anemia does not occur, and failure to thrive is the most common sign (99). The phenotypic expression of all variants of homocystinuria may vary substantially, even within the same family (99). The effect of treatment with cobalamin, folic acid, and/or betaine is variable, but may be successful if the treatment is initiated early (99).

tHcy in diagnosis of homocystinuria. Except in close family members of a homocystinuria patient, hyperhomocysteinemia is rarely explained by the genetic defects causing homocystinuria. However, in individuals with increased tHcy >100 μmol/L, homocystinuria should be suspected. Milder variants may produce lower tHcy concentrations. High methionine (10–50 times normal) combined with low cystathionine is indicative of CBS deficiency; low or normal methionine combined with increased cystathionine suggests a Hcy remethylation defect (70). Those

with MTHFR deficiency have red cell folate concentrations within reference values, whereas those with methionine synthase deficiency or defects in intracellular cobalamin metabolism have low red cell folate and a megaloblastic anemia *(99)*. Marked increases in both tHcy and MMA are found with some defects in cobalamin transport and metabolism *(99)*.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- tHcy should be measured in children/young adults with unexplained thrombotic disease, failure to thrive, mental retardation, psychiatric disease, lens dislocation, progressive myopia, or connective tissue disorders.
- Homocystinuria should be suspected in any person with severe hyperhomocysteinemia that is not explained by vitamin deficiency or renal failure.
- In homocystinuria, the amino acid pattern, blood vitamin concentrations, and MMA will reveal the likely site of the defect, but the genetic cause should, if possible, be identified.
- Treatment and follow-up of homocystinuria patients depend on the metabolic defect.
- Siblings of homocystinurics should be tested.
- Heterozygosity for CBS deficiency rarely leads to marked changes in tHcy or related metabolites. Identification of the carrier state requires DNA analysis.

RECOMMENDATIONS IN THE RESEARCH SETTING

- In addition to tHcy, the related metabolites (e.g., methionine, cystathionine, cysteine, and MMA) and the relevant vitamin should be measured. If possible, the genetic defect should be identified.
- In CBS heterozygotes, who often have normal fasting tHcy concentrations, measurement of tHcy in urine or after methionine loading may reveal abnormal Hcy metabolism.

FOLATE AND COBALAMIN DEFICIENCIES

Target populations. The target population includes individuals with clinical symptoms suggestive of folate or cobalamin deficiency and individuals who are at risk of developing a deficiency (Table 7 and Fig. 6).

Background. Folate deficiency occurs at all ages and is usually a result of poor diet, malabsorption, alcoholism, or use of certain drugs *(108)*. It is common during pregnancy *(108)*. The prevalence in US adults was ~20% before folic acid fortification, but it is now <2% *(76)*. Cobalamin deficiency is most often observed in the elderly (prevalence ~10–15%), where it is nearly always attributable to malabsorption caused by lack of intrinsic

factor (pernicious anemia), gastric atrophy, or ileal disease *(109)*. Newborns frequently have low cobalamin (see below) *(110)*. Age-independent causes include inadequate intake (e.g., vegetarians) or use of certain drugs (Table 4).

Diagnosis of folate or cobalamin deficiency is not straightforward. The definition of deficiency is debated, and there are no gold standards for diagnosis *(2, 108, 109)*. Megaloblastic changes are a late event in the disease process *(111)*, and neuropsychiatric symptoms may be present without hematologic findings *(109, 112)*. Furthermore, chronic, low-normal vitamin concentrations are related to serious conditions such as CVD, cancer, pregnancy complications, birth defects, psychiatric disorders, and cognitive impairment *(113–115)*. Hence, the aim is increasingly for early diagnosis in a preclinical state.

The basis for use of tHcy and MMA measurements in assessment of vitamin status is because these metabolites begin to increase sharply at low-normal concentrations of cobalamin (MMA and tHcy) and folate (tHcy only; Fig. 7) *(50, 116–119)*. Increased MMA or tHcy most frequently results from impaired vitamin function, and the metabolite concentrations will usually return to normal within 1 or 2 weeks after appropriate vitamin treatment *(82, 120, 121)*.

Comparison of blood tests. No single blood test used for assessment of vitamin status provides certain diagnosis, but each has advantages and limitations *(2, 121)*. Below we discuss folate, cobalamin, MMA, and tHcy measurements. For information on other tests used in the diagnosis of vitamin deficiency (e.g., blood and bone marrow examinations, serum gastrin, autoantibodies, Schilling test, and deoxyuridine suppression test), readers are referred to specialized reviews *(2, 121)*.

Blood vitamin measurements are established and simple to perform, but are increasingly considered to be neither sufficiently sensitive nor specific *(2, 122–125)*. There is limited consensus on reference limits and desirable concentrations *(108, 109)*. In addition, folate measurements, in particular red cell folate, are hampered by low analytical performance *(2)*. These factors may explain why up to 50% of the individuals with low vitamin concentrations have no other biochemical or clinical finding and why patients with low-normal vitamin concentrations may have symptoms that respond to vitamin treatment *(108, 109, 121, 126, 127)*. New assays for holotranscobalamin (holoTC) have now become available *(128, 129)*. Low holoTC concentrations could be an early marker of cobalamin deficiency *(111)*; recent studies show promising results *(130–133)*, but the clinical utility of this test remains to be documented.

Among the metabolites, MMA is often deemed superior to tHcy in relation to cobalamin deficiency *(134, 135)* because it is more specific and less susceptible to preanalytical errors than tHcy. In a person with normal renal function and low cobalamin, MMA concentrations above the threshold are often seen as proof of disturbed cobal-
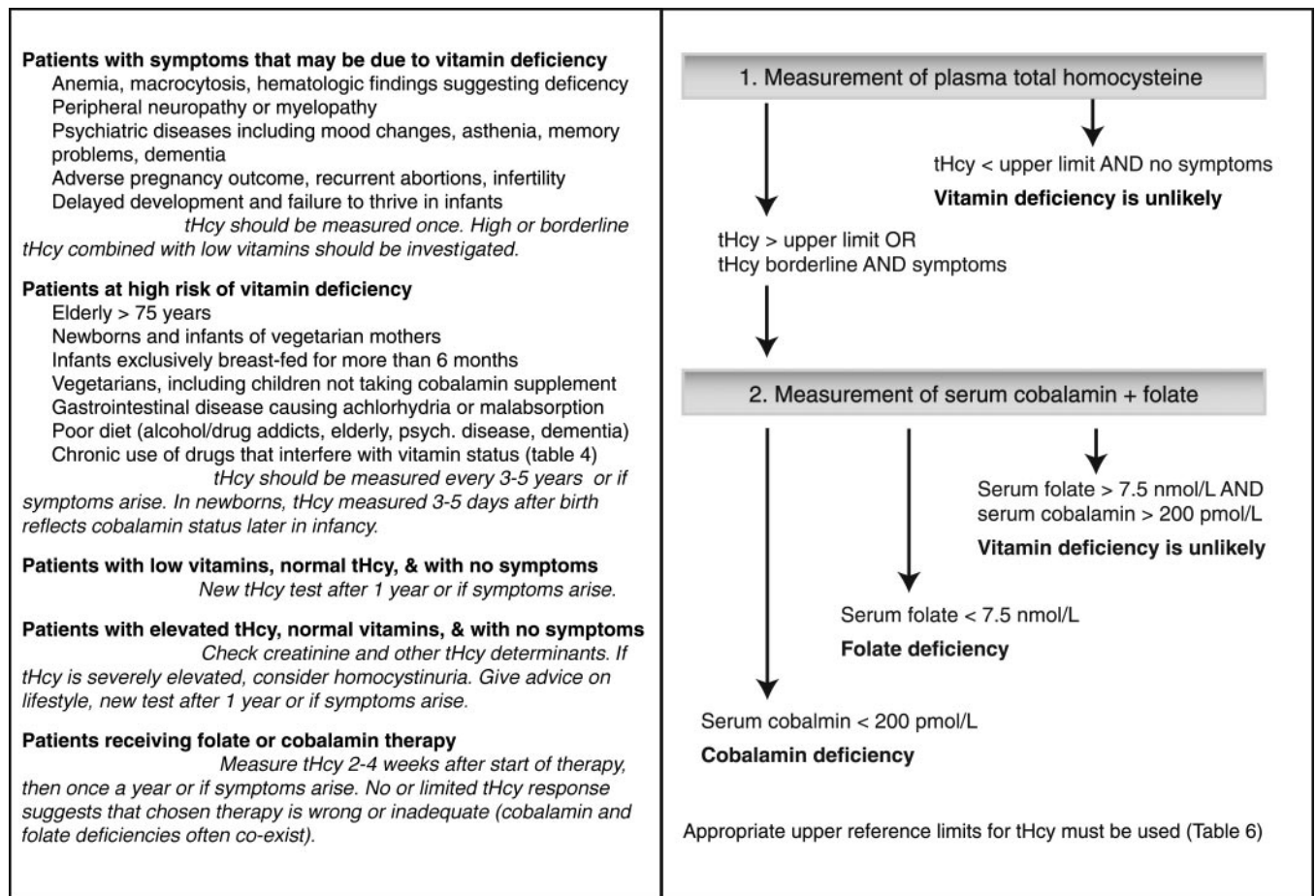
Patients with symptoms that may be due to vitamin deficiency
   Anemia, macrocytosis, hematologic findings suggesting deficiency
   Peripheral neuropathy or myelopathy
   Psychiatric diseases including mood changes, asthenia, memory problems, dementia
   Adverse pregnancy outcome, recurrent abortions, infertility
   Delayed development and failure to thrive in infants
            tHcy should be measured once. High or borderline tHcy combined with low vitamins should be investigated.

Patients at high risk of vitamin deficiency
   Elderly > 75 years
   Newborns and infants of vegetarian mothers
   Infants exclusively breast-fed for more than 6 months
   Vegetarians, including children not taking cobalamin supplement
   Gastrointestinal disease causing achlorhydria or malabsorption
   Poor diet (alcohol/drug addicts, elderly, psych. disease, dementia)
   Chronic use of drugs that interfere with vitamin status (table 4)
            tHcy should be measured every 3-5 years or if symptoms arise. In newborns, tHcy measured 3-5 days after birth reflects cobalamin status later in infancy.

Patients with low vitamins, normal tHcy, & with no symptoms
            New tHcy test after 1 year or if symptoms arise.

Patients with elevated tHcy, normal vitamins, & with no symptoms
            Check creatinine and other tHcy determinants. If tHcy is severely elevated, consider homocystinuria. Give advice on lifestyle, new test after 1 year or if symptoms arise.

Patients receiving folate or cobalamin therapy
            Measure tHcy 2-4 weeks after start of therapy, then once a year or if symptoms arise. No or limited tHcy response suggests that chosen therapy is wrong or inadequate (cobalamin and folate deficiencies often co-exist).

1. Measurement of plasma total homocysteine

tHcy < upper limit AND no symptoms
Vitamin deficiency is unlikely

tHcy > upper limit OR
tHcy borderline AND symptoms

2. Measurement of serum cobalamin + folate

Serum folate > 7.5 nmol/L AND
serum cobalamin > 200 pmol/L
Vitamin deficiency is unlikely

Serum folate < 7.5 nmol/L
Folate deficiency

Serum cobalmin < 200 pmol/L
Cobalamin deficiency

Appropriate upper reference limits for tHcy must be used (Table 6)

Fig. 6. Use of tHcy measurements in the diagnosis of folate and cobalamin deficiencies.
(*Left*), target populations and testing recommendations for use of tHcy in vitamin diagnosis. (*Right*), suggested algorithm for use of tHcy as a routine screening test in individuals at high risk of deficiency or as an initial test in individuals with few, or only nonspecific, signs or symptoms. In patients with findings strongly indicative of folate/cobalamin deficiency, such as megaloblastic anemia, blood concentrations of folate and cobalamin should be measured first. *Psych.*, psychiatric.

amin function (1, 118). A disadvantage, however, is that MMA measurements are expensive and not widely available (2). Some data suggest that increased MMA predicts neither symptoms of deficiency nor response to cobalamin treatment (136–138).

The argument against the use of tHcy measurements is that it is not a specific test because the concentration is influenced by numerous factors (Tables 3 and 4). However, most of these factors change tHcy concentrations through their effect on folate or cobalamin status. In addition, a large proportion of folate and cobalamin measurements are used as screening tests or in relation to nonspecific symptoms that can be explained by a deficiency of either vitamin (118, 138). Thus, as a screening test, tHcy is probably superior to MMA because tHcy measurements are less expensive, widely available, and reflect both cobalamin and folate status.

The diagnostic utility of tHcy and MMA in vitamin assessment is well documented, and interested readers are referred to published reports (1, 49, 82, 118, 120, 135) and reviews (108, 109, 121). Unfortunately, few studies have actually compared the sensitivity or specificity of these metabolites with folate and cobalamin measurements in relation to clinical outcome. With the lack of gold standards for diagnosis, optimal design of such studies requires that the inclusion criterion should be a finding, symptom, or disease suggesting vitamin deficiency but identified by means other than vitamin or metabolite measurements. An alternative approach is to use a large cohort with sufficient clinical or hematologic data (see Fig. 8).

tHcy in assessment of vitamin status. Despite the limitations of the published studies, the evidence suggests that an increased tHcy or MMA combined with a low vitamin concentration is better than either alone to identify individuals with symptoms (Fig. 8) (1, 53, 54, 125) and those who would benefit from treatment (Fig. 9) (49, 82, 127, 139). High tHcy or MMA concentrations may also help to identify individuals at risk of developing adverse effects from drugs that interfere with folate or cobalamin status (140–142).
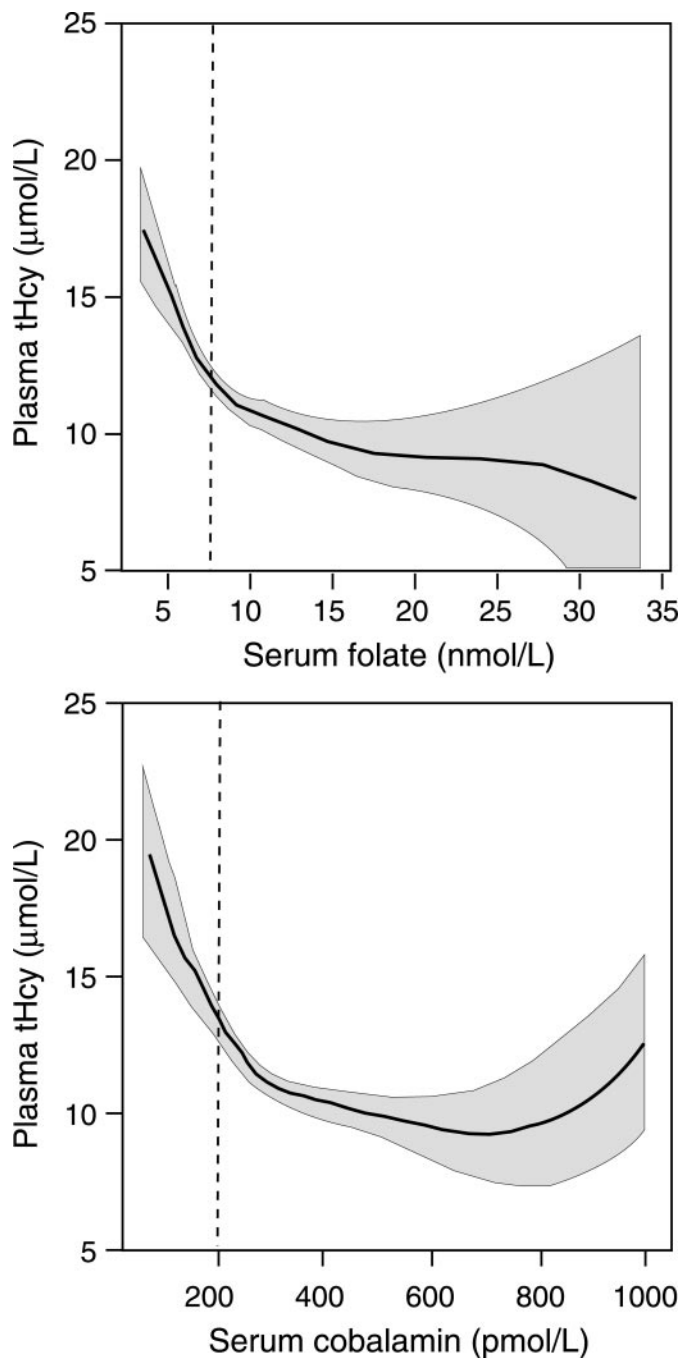
Fig. 7. Dose–response relationship between vitamin concentrations and plasma tHcy in healthy adults.

Plasma tHcy begins to increase at low-normal concentrations of both vitamins. Lower reference limits, 150 pmol/L for cobalamin and 5 nmol/L for folate. *Vertical lines* indicate recommended lower action thresholds for cobalamin and folate (see Fig. 6). The figure has been modified from Nygård et al., 1999 *(288)* with permission by the *Journal of Internal Medicine*.

tHcy measurements are increasingly being used to screen for vitamin deficiency in the general or high-risk populations *(53, 118, 121, 126, 138)*. Such studies have used tHcy thresholds consistent with the upper reference limits listed in Table 6, i.e., corresponding to the 95th–97.5th percentiles in a presumed healthy population. The

vitamin thresholds used for screening are usually 200–250 pmol/L for cobalamin *(2, 118, 126)* and 7–10 nmol/L for serum folate *(50, 53, 108)*. These thresholds fit with the concentrations at which tHcy begins to increase steeply (Fig. 7), but are substantially higher than the statistical lower reference limits for cobalamin and for folate in nonfortified populations *(108, 109)*.

It is usually suggested that vitamin measurements should precede tHcy (or MMA) determination *(2, 118)*. However, in patients with no or only diffuse symptoms, tHcy measurements are probably superior primary screening tests (Figs. 8 and 9) *(53, 126, 138)*.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Diagnosis of folate/cobalamin deficiency requires either typical symptoms accompanied by low vitamin concentrations or the combination of increased tHcy (or MMA) with low vitamin concentrations.
- For biochemical assessment of vitamin status, the least expensive approach is to measure tHcy and vitamins sequentially, either starting with tHcy (see Fig. 6) followed by the vitamins, or vice versa.
- In patients with no or vague symptoms, tHcy measurements are probably better screening tests than vitamin measurements to identify patients in need of further medical attention.
- tHcy (or MMA) should be used to monitor treatment response after vitamin supplementation.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Studies on folate or cobalamin status should include tHcy, creatinine, and both vitamins.
- Data on holoTC, vitamins B_2 and B_6, metabolites (e.g., MMA and sulfur amino acids), and relevant genetic and environmental determinants (Tables 3 and 4) could enhance the quality of the study.
- Comparing the ability of the different tests to identify patients with signs or symptoms and/or patients responding to treatment could enhance the clinical usefulness of these studies.
- Prospective studies with long follow-up times should include repeat measurements some years after the initial measurement in all or in a random sample to correct for the regression dilution bias.

CVD

Target populations. The target populations include individuals with established, or at high-risk of developing, arterial or venous occlusive vascular disease.

Background. Homocystinuria, irrespective of the enzymatic defect, leads to severely increased tHcy and a high incidence of arterial and venous thromboembolic events
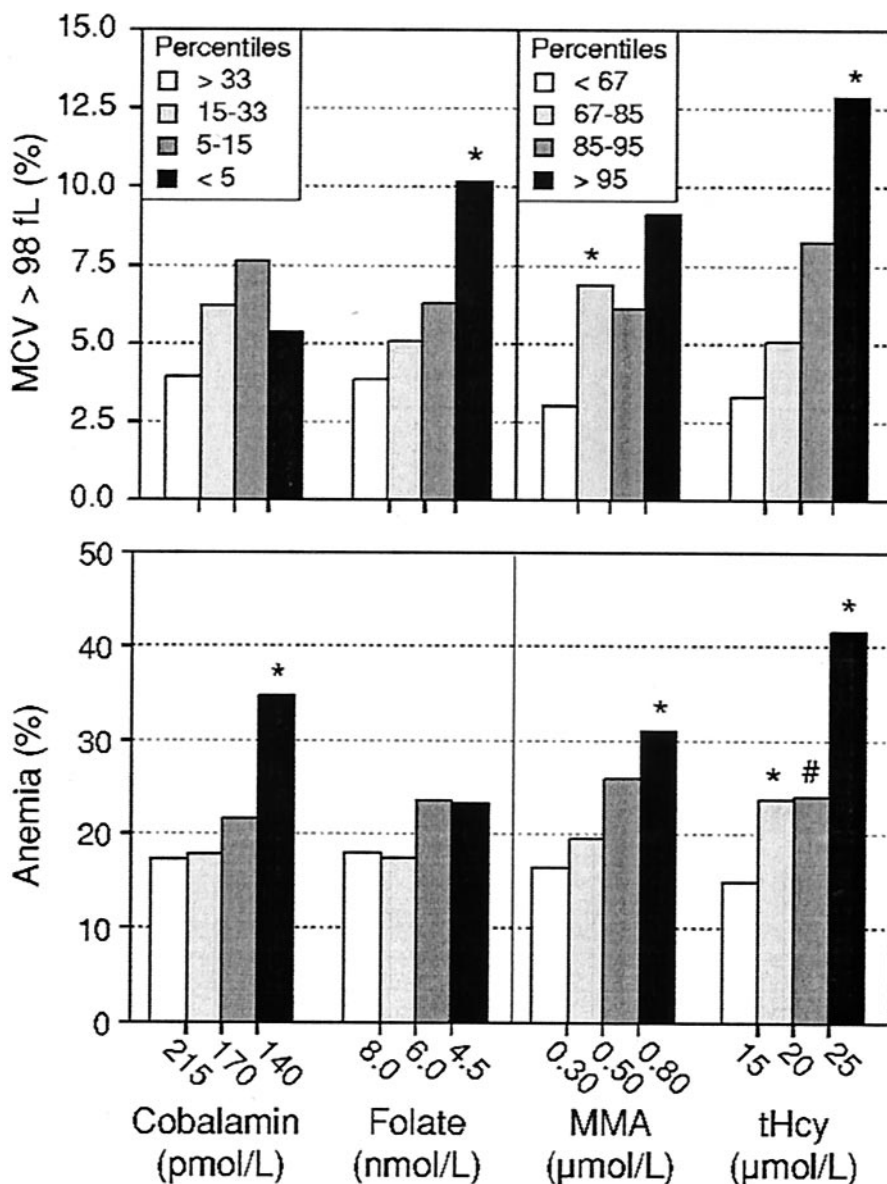
Fig. 8. Ability of low folate or cobalamin or of increased MMA or tHcy to identify elderly individuals with macrocytosis or anemia.

Data are from the UK-based OHAP Study, which includes 1502 free-living elderly individuals (age range, 67–102 years) *(53)*. Cobalamin, folate, MMA, and tHcy were divided into four categories with similar lower or respective upper thresholds for the vitamins (~33rd, 15th, and 5th percentiles) and the metabolites (~67th, 85th, and 95th percentiles). Vitamin concentrations above the ~33rd percentile or metabolite concentrations below the ~67th percentile are considered normal. The next two categories represent borderline ranges, whereas vitamin concentrations below the ~5th percentile and metabolites above the 95th percentile are clearly abnormal. It can be seen that increased tHcy or MMA concentrations are superior to low vitamin concentrations in identifying individuals with hematologic findings. ∗, $P < 0.05$ compared with those with normal vitamin or metabolite concentrations; #, $P = 0.051$ compared with those with normal tHcy concentrations.

(70, 99). These patients also have vascular changes, and this observation led McCully in 1969 *(143)* to propose his Hcy theory of atherosclerosis in the general population. Since then, the association between tHcy and CVD has been documented in many epidemiologic studies (for details see Refs. *(4, 144, 145)*. Moderately increased tHcy is related to both venous and arterial occlusive disease *(3)*. The relationship between tHcy and CVD is dose dependent and independent of other risk factors *(3, 4)*. Data from knock-out mice support the view that increased Hcy causes vascular disease *(146, 147)*. In humans, the deleterious effects of Hcy on endothelial and vascular function and blood coagulation provide pathophysiologic explanations for the increased CVD risk in hyperhomocysteinemia *(3, 148, 149)*.

Nevertheless, for three reasons there has been debate about the causal role of tHcy, and, hence, its clinical

significance in CVD *(150)*: (*a*) the MTHFR 677C→T polymorphism, which is a strong risk factor for increased tHcy but not for CVD; (*b*) the apparent discrepancy between prospective and retrospective case–control studies; and (*c*) the lack of data from controlled clinical trials.

The apparent lack of MTHFR 677C→T effect *(57)* is related to the low power of most studies *(150)*. In a recent metaanalysis including 11 000 CVD cases and 13 000 controls, the TT genotype was associated with a 16% increase in CVD risk *(58)*. This risk enhancement corresponds to the extent of tHcy increase observed in individuals with the TT genotype *(4, 58, 150)*. Thus, the results for individuals with the MTHFR 677TT genotype support the hypothesis that Hcy is causally related to CVD *(58)*.

The discrepancy between prospective and retrospective case–control studies *(4, 144, 151)* arises in part because most prospective studies start with a healthy pop-
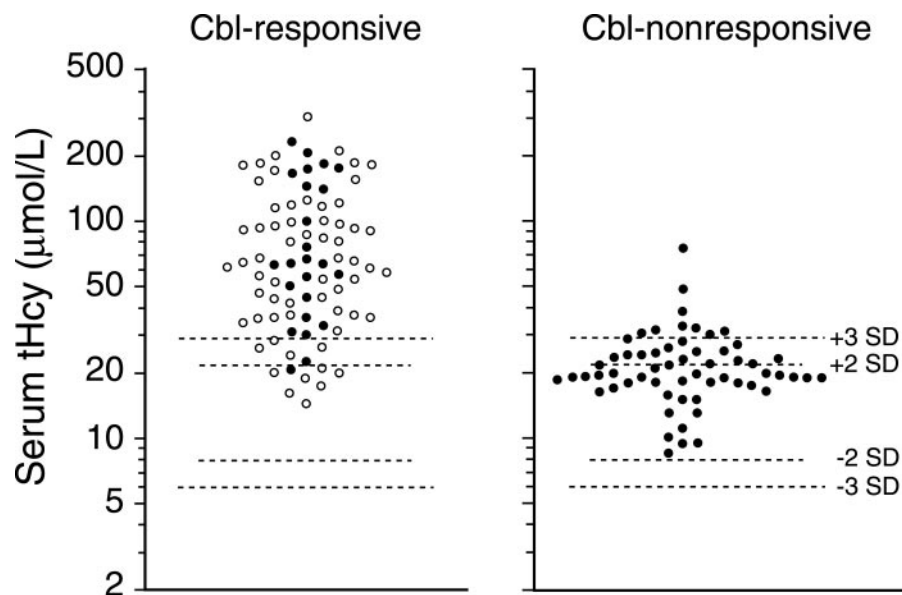
Fig. 9. Pretreatment serum tHcy concentrations in cobalamin-deficient patients according to clinical and hematologic responses to cobalamin (*Cbl*) treatment.

(*Left*), pretreatment serum tHcy concentrations in 86 patients who experienced significant hematologic responses or improvement of neuropsychiatric symptoms. ●, patients who also showed an improvement in neuropsychiatric symptoms. (*Right*), pretreatment serum tHcy concentrations in 59 patients who did not respond to vitamin therapy. Those with increased tHcy among the nonresponders were frequently preclinical cases of pernicious anemia (not shown). *Horizontal lines* represent mean ± 2 or 3 SD in a control population. Data are from Stabler et al., 1990 *(49)*, and the figure has been modified with permission by the American Society of Hematology.

ulation. In such studies, tHcy is a modest predictor of CVD events. In two recent large-scale metaanalyses, a 25% (3 μmol/L) reduction in tHcy in population-based cohort studies was associated with a 11–16% decrease in the risk of ischemic heart disease, 19–22% decrease in the risk of stroke and 25% decrease in the risk of deep venous thrombosis *(4, 145)*. In contrast, increased tHcy is a strong risk factor for CVD events and mortality in patients with coronary artery disease (Fig. 10), diabetes, renal failure, and systemic lupus erythematosus *(152–156)*. Hence, irrespective of causality, increased tHcy is a prognostic factor in certain groups with a high CVD risk profile.

Results from intervention trials with B vitamins are sparse. Some studies show that tHcy-lowering therapy slows the progression of coronary and peripheral atherosclerosis *(157–159)*. However, a recent study found that patients receiving folic acid, cobalamin, and vitamin B$_6$ in combination had increased risk of in-stent restenosis *(160)*. Many of the ongoing trials will lack power as a result of the folic acid fortification program *(161)*. Thus, we may only know the public health implications of B-vitamin intervention by performing a metaanalysis of results from ongoing CVD trials.

tHcy in CVD risk assessment. Epidemiologic data suggest that tHcy screening in the general population is not justified *(4, 144, 162)*. However, in high-risk populations, increased tHcy is a prognostic marker for an increased risk of new CVD events or mortality *(150)*. Whether this reflects a causal role of tHcy may be answered by the ongoing clinical trials *(163)*. The outcome of the trials will certainly influence recommendations for the use of tHcy in CVD risk assessment.

The question about desirable tHcy concentrations is being debated. There is a dose–response relationship between tHcy and CVD risk from low concentrations up to at least 20 μmol/L *(3, 152, 164)*. Some experts suggest that tHcy should be <10 μmol/L *(20, 81)*. With such a cutoff, 30–50% of the general population would be defined as "hyperhomocysteinemic"; this proportion would be higher among CVD patients and the elderly. A more realistic target can be proposed from prospective studies; a significant and substantial increase in CVD risk is observed above 13–15 μmol/L *(144, 152, 156, 164–167)*. Firm recommendations about desirable tHcy concentrations will be available only after the results of clinical trials are reported and may depend on whether clear thresholds for risk reduction after vitamin intervention are demonstrated.

Overall, the use of tHcy in CVD risk assessment is controversial in relation to both how and when to use the test and what to do about the result. Within our group, we were unable to reach a consensus, and the recommendations listed below for the clinical setting reflect the majority view.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Measurement of tHcy in the general population to screen for CVD risk is not recommended.
- In young CVD patients (<40 years), tHcy should be measured to exclude homocystinuria.
- In patients with CVD or persons with high risk of CVD events, a high tHcy concentration should be used as a prognostic factor for CVD events and mortality.
- CVD patients with tHcy >15 μmol/L belong to a high-risk group; it is especially important for them to follow a healthy lifestyle and to receive optimal treatments for known causal risk factors.
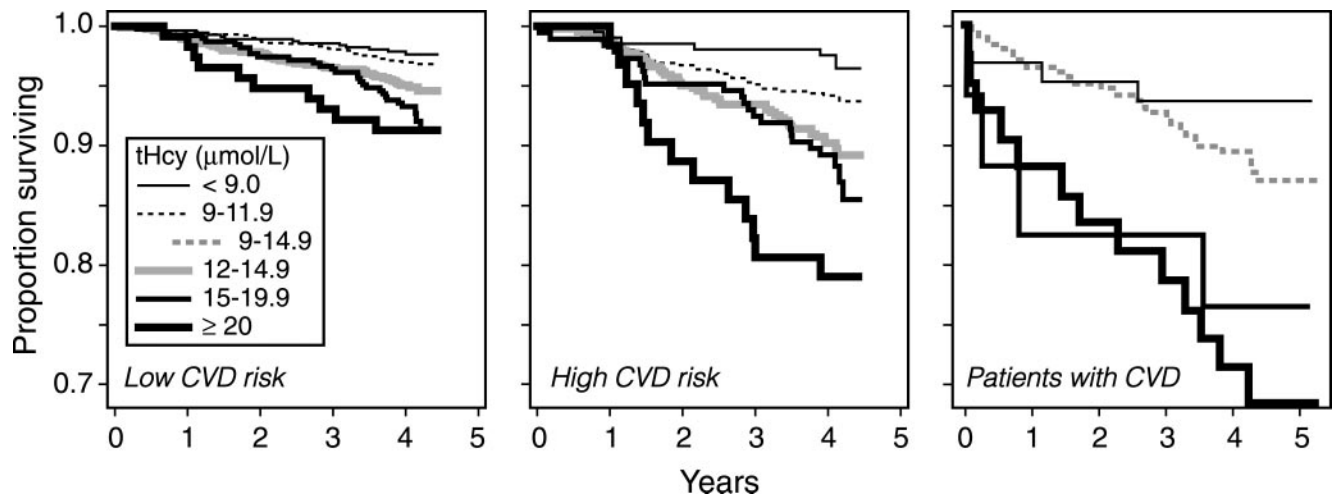
Fig. 10. Prediction of mortality by plasma tHcy, depicted as Kaplan–Meier survival curves, in three groups characterized by different CVD risk profiles.

Shown are survival at five (*left* and *middle*) or four (*right*) different tHcy concentrations. The *curves* have been estimated by a stratified Cox regression analysis. *Low CVD risk*, individuals in the Hordaland Homocysteine Study who at inclusion reported no history of CVD, hypertension, or diabetes. *High CVD risk*, individuals in the Hordaland Homocysteine Study who had a history of CVD, hypertension, or diabetes. *Patients with CVD*, patients from Hordaland County with angiographically confirmed coronary heart disease. Data are from Nygård et al., 1997 *(242)* and Vollset et al., 2001 *(164)*. The *left* and *middle panels* are modified from Vollset et al., 2001 *(164)*, with permission of the *American Journal of Clinical Nutrition* © Am. J. Clin. Nutr. American Society for Clinical Nutrition.

- Increased tHcy combined with low vitamin concentrations (Fig. 6) should be handled as a potential vitamin deficiency. Other causes of increased tHcy (Tables 3 and 4) should be considered.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Measurement of tHcy should be considered in clinical and epidemiologic studies on CVD when the conventional risk factors are being assessed.
- In studies that examine the relationship between tHcy and CVD, blood concentrations of folate, cobalamin, and creatinine should also be analyzed.
- See also the recommendations listed for research on folate and cobalamin deficiencies.

RENAL FAILURE

Target populations. The target populations include patients with impaired renal function, in particular dialysis patients.

Background. There is an inverse relationship between tHcy and renal function, ranging from normal renal function to end stage renal disease. Most dialysis patients (>85%) have hyperhomocysteinemia *(68)*. Folic acid supplementation efficiently reduces tHcy in patients with renal disease, but higher doses are required, and the tHcy concentrations usually remain increased even after optimal treatment *(68, 168)*.

CVD is a major cause of death in renal patients *(68)*. Prospective studies have demonstrated that tHcy concentrations predict both CVD morbidity and mortality, par-

ticularly in individuals with tHcy >30 μmol/L *(155, 169–171)*. Increased tHcy is also related to increased risk of hemodialysis access thrombosis, a common complication in dialysis patients *(172)*.

tHcy in risk assessment. There are no data for clinical outcomes from intervention trials, and evidence that reductions in tHcy have a beneficial effect is therefore lacking. However, as for CVD, tHcy measurements can be used as prognostic factors for CVD events and mortality.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Measurement of tHcy in patients with renal disease is controversial, but a high tHcy (>30 μmol/L) may be used as a prognostic factor for mortality and CVD events.
- Recommendations for treatment of renal patients with mild hyperhomocysteinemia cannot be justified until the results of clinical trials are known.
- Unexplained mild or intermediate hyperhomocysteinemia that is not explained by folate or cobalamin status should prompt an assessment of renal function.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Research on tHcy in the general population should include markers of renal function.
- Measurement of tHcy should be considered in clinical and epidemiologic studies on renal disease when the conventional risk factors are being assessed.

- In studies that examine the relationship between tHcy and renal disease, blood concentrations of folate and cobalamin should also be analyzed.
- See also the recommendations listed for research on folate and cobalamin deficiencies.

PSYCHIATRIC DISORDERS AND COGNITIVE IMPAIRMENT

Target populations. Target populations include patients, including children, with psychiatric diseases or cognitive impairment as well as the elderly, especially those >75 years.

Background. Pernicious anemia is associated with psychiatric disease, including psychosis, depression, paranoia, violent behavior, and changes in personality. However, pernicious anemia is not a common cause of these conditions *(173)*. There are many reports of an association between folate, cobalamin, tHcy, and MMA and depression *(174–176)*, in particular in the elderly *(138, 177, 178)*. The response to antidepressant drug treatment is often poorer in patients with low folate status *(179)*, and improved clinical responses can sometimes be obtained by combining drug treatments with folic acid supplementation *(180)*. An association between schizophrenia and increased tHcy has been described *(181)*.

Cobalamin and folate are required for normal cognitive function *(182)*, and there is an inverse relationship between cognitive scores and B-vitamin and tHcy concentrations in healthy elderly *(183–187)*. Pernicious anemia is associated with cognitive impairment, including loss of concentration and memory, disorientation, and dementia, with or without mood changes *(173)*. Patients with Alzheimer disease, vascular dementia, or white matter disease often have low concentrations of these vitamins and/or high tHcy concentrations *(7, 8, 133, 188–193)*. The association between tHcy and Alzheimer disease is dose dependent and even stronger in those with histopathologically confirmed diagnosis *(192)*. Increased tHcy or low folate or cobalamin precedes cognitive decline *(194–196)*: Hyperhomocysteinemic individuals have an increased rate of atrophy of the medial temporal lobe *(192)* and a more rapid decrease in cognitive test scores *(194)*. A large prospective study showed that increased tHcy up to 11 years before the diagnosis of dementia is associated with twice the risk of Alzheimer disease *(196)*. There are no randomized trials that have assessed the effects of tHcy-lowering therapy on cognitive function.

tHcy in risk assessment of psychiatric diseases. Despite increasing epidemiologic evidence, it is uncertain whether vitamin deficiency and hyperhomocysteinemia are frequent and treatable causes of dementia and psychiatric diseases *(109)*. However, in view of the importance of the B vitamins for brain function, assessment of vitamin status should always be done in patients with cognitive impairment or psychiatric disease, and tHcy measurements will therefore play an inherent role in assessment of B-vitamin status (see the section on folate and cobalamin deficiencies).

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Patients with psychiatric disorders, cognitive impairment, or dementia should have an assessment of cobalamin and folate status (see Fig. 6).
- Psychiatric and cognitively impaired patients with biochemical evidence of cobalamin and folate deficiencies should receive appropriate treatment because these deficiencies could be either the sole or contributory causes of the disorder.
- Psychiatric or cognitively impaired patients should be assessed for vitamin status every 3–5 years because changes in diet and/or drug treatments may lead to such deficiencies. Symptoms attributable to a deficiency may be erroneously attributed to the illness and therefore go undetected.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Studies on psychiatric disorders should include the measurement of tHcy if blood indices are considered as risk factors.
- In studies that examine the relationship between tHcy and psychiatric disease, blood concentrations of folate, cobalamin, and creatinine should also be analyzed.
- See also the recommendations listed for research on folate and cobalamin deficiencies.

PREGNANCY COMPLICATIONS AND BIRTH DEFECTS

Target populations. Target populations include women of childbearing age with, or at risk of, folate or cobalamin deficiency as well as women who have had a child with a birth defect or who have experienced pregnancy complications.

Background. Pregnant women have lower plasma tHcy than nonpregnant women *(197)*. The mean tHcy concentration in pregnant women is ~5–6 μmol/L, and tHcy concentrations >10 μmol/L are rarely observed *(66, 67, 198)*. As with nonpregnant women, tHcy is influenced by cobalamin and folate status *(66, 199, 200)*.

Increased tHcy is associated with an increased risk of a placental vasculopathy *(5, 201)*, which in turn is related to preeclampsia, recurrent early pregnancy loss, premature delivery, low birth weight, and placental abruption or infarction. These events have been related to fasting and

post-methionine-load tHcy concentrations (5, 6, 202–204). In most studies, tHcy has been measured either in the nonpregnant state (retrospectively) or at the time of the event. In two studies in which blood samples were collected before conception, increased tHcy was associated with increased risk of spontaneous abortions and preterm births (205, 206). The data on the predictive role of increased tHcy during pregnancy and the effect of tHcy-reducing therapy in the prevention of adverse pregnancy outcomes are conflicting (198, 207–209).

Maternal hyperhomocysteinemia is related to birth defects, including NTDs, orofacial clefts, clubfoot (6, 210, 211), and Down syndrome (212). Folic acid supplements in the periconceptual period and the first few weeks of pregnancy reduce the risk of NTDs (213–215). For this reason, all women of childbearing age should have a folate intake of at least 400 μg/day. Despite educational programs, women are often not aware of the benefits of folate (216). Many pregnancies are not planned, and women often do not know that they are pregnant at the critical period in relation to NTDs (217). Because of this, the US Food and Nutrition Board decided that folic acid fortification should be mandatory from 1998, and folic acid was added to the food supply between March 1996 and January 1998 (161). Fortification markedly improved folate status and may have reduced the NTD incidence by 20–40%, but it is uncertain whether this is attributable to the fortification because the downward trend started before fortification was introduced (218, 219). In regions with high NTD prevalence, such as China, the beneficial effect of increased folic acid intake has been substantial (220).

There are biologically plausible reasons that increased tHcy may be related to adverse pregnancy outcomes. Increased tHcy may directly or indirectly cause endothelial dysfunction, impair neurolation, reduce microfilament synthesis, inhibit DNA methylation and alter gene expression, and reduce S-adenosylmethionine-dependent methylation reactions (211). In addition to low folate and increased tHcy, low methionine concentrations and the MTHFR 677TT genotype are risk factors for pregnancy complications, birth defects, and Down syndrome (211, 212, 221, 222). Cobalamin status may also be important (223, 224), particularly in developing countries, where cobalamin deficiency is common (110, 200, 225).

tHcy in risk assessment. Increased tHcy is associated with birth defects and pregnancy complications, but it is uncertain whether tHcy-lowering treatment is beneficial in hyperhomocysteinemic women. The results of prospective studies on whether increased tHcy predicts adverse pregnancy outcomes are conflicting (198, 205–209). Thus, the use of tHcy measurement should be confined to certain high-risk groups.

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- Routine measurement of tHcy before or during pregnancy is currently not recommended.
- tHcy should be measured in pregnant women who suffer from early-onset pregnancy complications.
- Women who have experienced pregnancy complications or had a child with birth defects should have their tHcy and vitamin concentrations measured 3 months after the end of the pregnancy.
- In women with or at high risk of vitamin deficiencies, tHcy should be measured regularly (Fig. 6), preferably before and once during pregnancy to ensure optimal vitamin status for mother and child.
- tHcy or vitamin measurements should not replace the recommendation that all women of childbearing age have a folic acid intake of at least 400 μg/day.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Studies on birth defects and pregnancy complications should consider including tHcy measurements if blood indices are investigated as risk factors.
- Such studies may benefit from collecting blood samples in both the pregnant and nonpregnant state and from the child.
- In studies that examine the relationship between tHcy and adverse pregnancy outcomes, blood concentrations of folate, cobalamin, and creatinine should also be measured.
- See also the recommendations listed for research on folate and cobalamin deficiencies.

ROUTINE SCREENING

The term "routine screening" refers to automatic or repetitive testing in a defined population and usually involves testing of asymptomatic patients with the intention to discover subclinical disease (226). To be appropriate for screening, (a) a disease should be serious, (b) morbidity or mortality should be reduced significantly by starting treatment before symptoms develop, (c) the prevalence of preclinical disease should be high enough among the population screened to justify the cost, (d) a suitable screening test must be available, and (e) the screening procedure should be acceptable to the patient and society (226). Documentation is available relative to screening of tHcy in the elderly for cobalamin deficiency and, possibly, in newborns for cobalamin deficiency and homocystinuria. Screening should also be considered in some other groups at high risk of developing cobalamin or folate deficiency (Fig. 6). Available data do not support tHcy as a screening test for CVD in the general population (144).

SCREENING FOR COBALAMIN DEFICIENCY IN THE ELDERLY

Target populations. Target population is the elderly, in particular those >75 years.

Background. Approximately 10–15% of the elderly have biochemical evidence of cobalamin deficiency *(53, 126, 173)*. The idea of periodic screening of elderly people to detect early stages of cobalamin deficiency is not new *(227, 228)*, but the introduction of folic acid fortification of flour makes it more relevant: a high intake of folate may prevent the development of anemia, thereby masking cobalamin deficiency, and perhaps enhance the progression of neurologic disease *(229)*. Data from the US *(218)* suggest that for every baby saved from NTDs, 1 million persons have to eat fortified food, and that among these, 10–15% will be in an age group at high risk of cobalamin deficiency.

The deficiency in the elderly is usually caused by food cobalamin malabsorption, a condition that develops slowly over years and often with few hematologic findings *(109, 173)*. However, elderly with low serum cobalamin frequently have subtle disturbances in erythropoiesis *(230, 231)*. The neurologic symptoms of cobalamin deficiency, including fatigue, memory problems, and sensory neuropathy, are often thought to reflect "normal aging" *(109)*. Increased tHcy and low cobalamin in the elderly are inversely correlated with measures of well-being *(232)*, and elderly with high tHcy may experience a more rapid physical decline *(233)*.

tHcy in risk assessment. Cobalamin deficiency in the elderly fulfills most criteria for screening, but the approach has not been defined. Use of the conventional lower reference limit for serum cobalamin (~150 pmol/L) may cause a substantial proportion of the elderly in need of therapy to be missed *(82, 121, 126)*. It has been suggested that a higher threshold (200–250 pmol/L) should be used *(118, 173, 234)*. Measuring tHcy in those with borderline cobalamin will then help to identify individuals with a deficiency *(49, 53)*. The alternative is to use tHcy as the initial screening test (Fig. 6), which is probably a more suitable test for identifying patients in need of additional medical attention (Figs. 8 and 9).

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- In view of the high prevalence of cobalamin deficiency in the elderly and the availability of efficient and safe therapies, we recommend that all individuals > 75 years be screened at 3- to 5-year intervals by the approach shown in Fig. 6.

RECOMMENDATIONS IN THE RESEARCH SETTING

- See the recommendations listed for research on folate and cobalamin deficiencies.

SCREENING FOR HOMOCYSTINURIA AND COBALAMIN DEFICIENCY IN THE NEWBORN

Target population. Target population includes newborns 3–5 days after birth.

Background on homocystinuria. Homocystinuria attributable to CBS deficiency may be far more common than previously anticipated *(101)*. Early treatment, preferably from infancy, can prevent serious and life-threatening complications *(100, 103)*.

tHcy in risk assessment for homocystinuria. Newborn screening for homocystinuria is routine in several countries and has usually been achieved by measuring methionine in blood spots. With the novel techniques for tHcy determination, such as microtiter-based enzyme immunoassays and MS-MS *(13, 235)*, it is possible to screen babies for tHcy rather than methionine. Determination of tHcy may also identify babies with homocystinuria attributable to Hcy remethylation defects *(99)*.

Background on cobalamin deficiency. Newborn screening for hyperhomocysteinemia allows early detection of the relatively common condition of neonatal cobalamin deficiency. Cobalamin deficiencies in babies are nearly always attributable to poor cobalamin status in the mother, who is usually asymptomatic *(99)*. On the basis of the adult lower reference limit for serum cobalamin (150 pmol/L), ~10–15% of newborns are vitamin deficient *(110, 236)*. These babies frequently have increased tHcy and MMA as well *(110, 236)*. The tHcy concentration in a baby is determined primarily by the tHcy concentration in the mother and by the cobalamin status of both mother and child *(110, 236, 237)*.

The symptoms of neonatal cobalamin deficiency become evident 4–6 months after birth *(110)*. Exclusively breastfed infants of vegetarian mothers are at particular risk *(110, 236)*. Failure to thrive and neurologic deficits are more common than megaloblastic anemia. Neonatal cobalamin deficiency is difficult to identify, and the diagnosis can easily be missed until severe symptoms develop, at which time the neurologic damage may become irreversible *(99, 110)*.

tHcy in risk assessment for cobalamin deficiency. A high tHcy concentration differentiates between babies with and without cobalamin deficiencies *(236)*. Among newborns with increased tHcy, ~20% have low cobalamin. Moreover, babies with high tHcy and/or low cobalamin at birth continue to have poor cobalamin status later in infancy *(110, 236)*. Thus, newborn screening may be an efficient way to identify babies at risk. During the first few weeks after birth, babies frequently develop high MMA concentrations (benign methylmalonic aciduria) *(110)*, which are only partly explained by low serum cobalamin. Hence, in infancy, the usefulness of MMA as a specific cobalamin marker is uncertain.

Table 8. Some examples of unresolved questions.

Methods and performance	To provide certified reference material
	To set up international procedures for standardization of the plasma tHcy assay
Sample collection and handling	To develop a simple procedure for stabilizing tHcy in plasma/serum before centrifugation
	To assess other biological fluids (e.g., urine, whole blood, blood spots)
Biological and pharmacologic factors influencing tHcy	To identify the relevant genetic polymorphisms and their gene–gene and gene–environment interactions
	To document the mechanism of the factors influencing tHcy
	To investigate the effect of commonly used drugs on tHcy
	To identify "new" determinants
Reference intervals vs desirable concentrations	To determine the appropriate population for establishing reference intervals
	To establish desirable tHcy concentrations for the various clinical conditions
Methionine loading	To provide data from prospective studies and intervention trials about the role of methionine loading in clinical diagnostics
	To determine the mechanism, if any, and to investigate the physiologic correlate of methionine loading
Vitamin deficiencies, including vitamins B_2 and B_6	To identify the vitamin-responsive diseases and symptoms beyond anemia and demyelinating disease, e.g., CVD, cancer, cognitive impairment, and so forth
	To identify the best marker (e.g., tHcy, cobalamin, folate, MMA, holoTC) for identifying individuals who will develop vitamin-responsive symptoms/diseases and the best marker for identifying those who will benefit from treatment
	To establish the "action limit" for vitamins and metabolites in vitamin-responsive conditions
	To assess the value of routine screening for cobalamin deficiency in all newborns, elderly, and other high-risk populations
Homocystinuria	To establish the birth prevalence of CBS deficiency and other causes of homocystinuria
	To evaluate the value of newborn screening for homocystinuria based on tHcy measurements
	To optimize and improve treatment for the various types of homocystinuria
Various conditions (e.g., CVD, psychiatric disorders, cognitive impairment, adverse pregnancy outcome, cancer, and other diseases)	To provide sufficient evidence from clinical and epidemiologic studies and intervention trials to reject or confirm a causal relation between increased tHcy and these conditions
	To identify the mechanisms

RECOMMENDATIONS IN THE ROUTINE CLINICAL SETTING

- The value of routine tHcy screening in all newborns is currently not documented.
- Babies with homocystinuric siblings or parents should be tested.
- tHcy should be measured in newborns at high risk of cobalamin deficiency (e.g., babies of vegetarian mothers) and in babies who have been exclusively breastfed for >6 months.
- The amino acid pattern and vitamin concentrations could reveal the likely cause of a high tHcy value.
- Newborn screening requires methods with high capacity and low volume requirements (e.g., blood spots). Precision is less critical. The microtiter-based enzyme immunoassay and MS-MS fulfill the criteria.

RECOMMENDATIONS IN THE RESEARCH SETTING

- Research on newborn screening for hyperhomocysteinemia should include the related vitamins, amino acids, and in the subset with high tHcy, relevant genetic polymorphisms and mutations.

- In studies on neonatal vitamin deficiency, both the mother and child should be investigated, preferably both at birth and later in infancy.
- In studies on homocystinuria, it may also be valuable to include siblings and parents.
- Comparing the ability of tHcy vs metabolites, vitamins, and gene variants to identify babies who have homocystinuria or cobalamin deficiency could enhance the clinical usefulness of these studies.

Concluding Remarks

Making recommendations for the use of tHcy has not been straightforward because the evidence is incomplete and because so many research questions remain unanswered (Table 8). However, even without complete information, the clinician and the laboratory scientist need advice on what to do in a given situation. Without hard evidence, one has to rely on expert opinion, which will often provoke debate. We hope that the opinions presented here will stimulate clinicians and laboratory scientists to perform new studies and thereby provide the evidence where it is currently lacking.

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