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Assessment of homocysteine status

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Summary: Plasma total homocysteine (tHcy) determination is used in the diagnosis of homocystinuria, in cobalamin and folate deficiency and in cardiovascular risk assessment. However, determination of tHcy includes many pitfalls which complicate the assessment of homocysteine status. In the present article, we review basic knowledge for a rational use of plasma tHcy in diagnostic as well as scientific work. The subjects dealt with are procedures for sample handling and processing, the principles of tHcy analyses, and genetic and acquired determinants of the plasma tHcy concentration.

Traditionally, homocysteine and/or its oxidized forms (Hcy) were primarily used in the diagnosis of homocystinuria, i.e. inborn errors of metabolism associated with extremely elevated levels of Hcy in plasma and urine. Today the level of total Hcy (tHcy) is mostly used as a marker of folate and cobalamin function and in cardiovascular risk assessment. In contrast to homocystinuria which can be diagnosed with qualitative assays, diagnosis of vitamin deficiencies and vascular risk assessment depend on sensitive tHcy methods with high precision. Such methods are now available, and several laboratories are about to introduce tHcy determination as a clinical routine. This review summarizes the basic knowledge required for the rational use of tHcy in epidemiological research, as well as in clinical chemistry.

CHEMISTRY AND TERMINOLOGY

Homocysteine refers to a defined chemical compound: it is a sulphur amino acid with a free thiol (sulfhydryl) group. The pK_a of the thiol group is ~8.5, and this makes it susceptible to oxidation at physiological pH. In this article, the abbreviation Hcy refers to both homocysteine itself (reduced Hcy) and its oxidized species. Only trace amounts (<0.3 μmol/L) of reduced Hcy can be detected in plasma (Ueland 1995), whereas most Hcy exists as various disulphide forms. About 70% is bound to albumin (protein-bound Hcy or Hcy-albumin mixed disulphide), whereas the remaining 30% exists as mixed disulphides with other thiols, and the cysteine-Hcy disulphide is the most abundant species. The sum of free and bound Hcy in plasma is denoted total Hcy, and abbreviated tHcy (Ueland 1995).

In an adult population, the normal plasma tHcy is 5–15 μmol/L, with a mean concentration of about 10 μmol/L. Hyperhomocysteinaemia is usually defined as a plasma tHcy

>15 μmol/L, and is denoted moderate (15–30 μmol/L), intermediate (30–100 μmol/L) or severe hyperhomocysteinemia (>100 μmol/L) (Kang et al 1992).

ANALYTICAL METHODS

The methods for tHcy determination have been reviewed previously (Ueland et al 1993), and will be described only briefly here. They can be categorized into five types: (1) enzymic assays, (2) gas chromatography–mass spectrometry, (3) assays based on precolumn derivatization, HPLC and fluorescence detection, (4) HPLC and electrochemical detection, and (5) assays based on liquid chromatography and post-column derivatization, including the amino acid analyser. Notably, an immunoassay for Hcy determination (Shipchandler and Moore 1995) based on a modification of a previously described enzymic assay (Refsum et al 1985) may soon be commercially available. The principles for derivatization, separation and detection vary markedly between the methods, but all assays include treatment of whole plasma/serum with a reductant. The Hcy disulphides are then quantitatively converted into reduced Hcy, i.e. to one chemical form, which can be directly quantified or derivatized (Ueland et al 1993).

Good correlation between tHcy determined in different laboratories and with different methods has been obtained (Ueland et al 1993), but the methods differ in equipment costs, the expertise necessary, automation, the performance of the method, and the possibility of simultaneously measuring other metabolites in plasma. Choice of method should therefore be based on the laboratory experience and resources as well as the expected clinical or scientific use of the assay. Precision and accuracy are of less importance when tHcy determination is used in the diagnosis of homocystinuria associated with severe elevation of the tHcy level. In contrast, cardiovascular risk assessment requires a method with high precision.

With more specialized techniques, it is possible to determine the various Hcy species in plasma (Ueland 1995). They are often more responsive than tHcy in relation to vitamin deficiency (Mansoor et al 1994), food intake (Guttormsen et al 1994), methionine (Mansoor et al 1992) or Hcy loading (Mansoor et al 1993). These assays are complicated, and should be reserved for mechanistic and kinetic studies.

PROCEDURES FOR SAMPLE COLLECTION AND PROCESSING

The collection of blood samples is a critical step in the determination of tHcy. Generally, it is recommended that the subject should be fasting. A small meal probably does not affect the plasma tHcy level (Ubbink et al 1992). A protein-rich meal, however, causes an increase in plasma tHcy of 15–20%, reaching a maximum 6–8 h after food intake (Guttormsen et al 1994). When collecting the blood sample, the posture of the subject should be taken into account, since more than 70% of tHcy in plasma is bound to albumin, and the albumin concentration is lower in the supine than the sitting position (Leppanen and Grasbeck 1988).

There is a time- and temperature-dependent release of Hcy from blood cells; at room temperature, plasma tHcy increases 5–15% per hour (Ueland et al 1993). The absolute increase is independent of plasma tHcy level. Thus, nonoptimal sample handling tends to reduce the difference between high and low tHcy levels. If immediate centrifugation is not

possible, this artificial increase is reduced by keeping whole blood on ice (Fiskerstrand et al 1993) or by collecting it into a tube containing a stabilizer such as fluoride (Ubbink et al 1992). This latter procedure is practical (Möller and Rasmussen 1995), but may interfere with the tHcy or other analytical assays. After removal of the blood cells, tHcy in serum/plasma is stable for at least 4 days at room temperature, for 2 weeks at 0–2°C, and for years when kept frozen at −20°C (Ueland et al 1993).

The importance of food intake, posture during sample collection, and sample processing is related to the accuracy and precision required for a particular application. A small artificial increase does not interfere with the diagnosis of homocystinuria or of a severe vitamin deficiency. However, in research, the correlation between tHcy concentrations and various factors (vitamins, creatinine, etc.) may be underestimated. In vascular risk assessment, an increase in the tHcy concentration of 4–5 μmol/L is associated with ~40–60% increase in estimated risk (Arnesen et al 1995; Boushey et al 1995). In such investigations, the blood samples should be collected under standardized conditions.

Both in whole blood and in plasma, there is a continuous redistribution between the various Hcy species, and within a few hours after blood collection (at room temperature) or after freezing and thawing the sample, the major proportion of Hcy is protein-bound. Reliable measurement of free Hcy or the other Hcy species in plasma therefore requires immediate (within seconds to minutes) deproteinization or sample processing. Determination of the various Hcy species is therefore impractical in the clinical routine (Ueland et al 1993; Ueland 1995).

METHIONINE AND HOMOCYSTEINE LOADING

The methionine loading test was originally introduced to detect heterozygosity for cystathionine β-synthase deficiency (Brenton et al 1966; Fowler et al 1971). Presently, it is used to stress the Hcy metabolizing pathways, and is often included in clinical studies on vascular disease. The procedure involves oral intake of a standard dose of meth-ionine (0.1 g/kg or 3.8 g/m^2), and tHcy is usually measured after a fixed time interval of 4 or 6 h (Ueland et al 1993). Recently, it was shown by Bostom and colleagues that the tHcy concentration in a sample collected after 2 h is highly correlated to the 4 h post-load value (Bostom et al 1995c), and this abbreviated form may be more practical in the clinical setting.

The post-load tHcy concentration is probably more sensitive than the fasting concentration to disturbances in the transsulphuration pathway. Thus, subjects with impaired ability to remethylate Hcy (cobalamin and folate deficiency) have hyperhomocysteinaemia during fasting, but may have a normal increase in tHcy after methionine loading. In contrast, subjects with a mild disturbance of the transsulphuration pathway often have a normal fasting tHcy concentration but are methionine intolerant (Brattström et al 1990), and plasma pyridoxal phosphate but not folate or cobalamin concentrations correlate with an abnormal methionine response (Verhoef et al 1997). Vitamin B_6-deficient subjects have normal fasting but abnormal post-load plasma tHcy concentration (Ubbink et al 1996b), and post-load but not fasting tHcy concentration is reduced in response to vitamin B_6 therapy. However, fasting tHcy concentrations are increased in subjects with severe inhibition of transsulphuration pathway, as observed in cystathionine β-synthase deficiency

(Mudd et al 1995). Thus, unequivocal identification of the site of the metabolic defect, based on fasting and post-load tHcy concentrations, is probably not feasible.

A frequently asked question is whether the methionine loading test is actually necessary. The fasting and post-load tHcy concentrations are significantly correlated: they discriminate between vascular patients and controls equally well, but the results do not completely overlap (Mansoor et al 1995). A recent study demonstrates that fasting tHcy alone fails to identify >40% of subjects with methionine intolerance (Bostom et al 1995b). Thus, determination of tHcy after methionine loading is probably a valuable adjunct to fasting tHcy, especially in cardiovascular risk assessment. It remains to be seen whether there is a physiological correlate, for example whether elevated post-load tHcy reflects a postprandial increase in tHcy.

It is also possible to perform peroral Hcy loading. L-Homocysteine thiolactone is used to prepare L-homocysteine, which is administered to the subject (65 μmol/kg). The elimination of Hcy from plasma can then be followed for the next 24–72 h (Guttormsen et al 1993). The plasma clearance of tHcy is about 100 ml/min, corresponding to an elimination half-life of 3–4 h (Guttormsen et al 1993). Notably, subjects with severe cobalamin/folate deficiency have normal tHcy clearance, suggesting that their hyperhomocysteinaemia is due to increased release of Hcy from the tissues to the plasma compartment (Guttormsen et al 1996b). In contrast, subjects with renal failure (Guttormsen et al 1995) have markedly reduced clearance. Conceivably, the Hcy loading test measures the Hcy elimination from plasma, whereas the methionine loading probably reflects intracellular Hcy formation and metabolism and the resulting Hcy egress from tissues to plasma. Comparison of the areas under the curves for methionine (Refsum et al 1989) and tHcy (Guttormsen et al 1993) suggest that only about 10% of methionine administered in the loading test is released to plasma as Hcy.

DETERMINANTS OF PLASMA TOTAL HOMOCYSTEINE

Women have lower tHcy concentrations than men, and tHcy increases with age. This may be due to differences in vitamin status between the sexes and in different age groups (Selhub et al 1993). The higher muscle mass in men may also explain the differences between the sexes. Formation of creatine from guanidinoacetate is dependent on adenosylmethionine; thus, for every molecule of creatine (and creatinine), one molecule of Hcy is formed (Brattström et al 1994; Mudd et al 1995).

Renal function has a central role in the elimination of tHcy from plasma (Bostom et al 1995a; Guttormsen et al 1995). Urinary excretion of Hcy, however, is very low (Refsum et al 1985; Guttormsen et al 1995), and extensive metabolism of Hcy is probably taking place in the kidneys (Bostom et al 1995a). The increase in tHcy seems to be more closely related to GFR than to the increase in serum creatinine (Chauveau et al 1993). Recently it has been demonstrated that GFR is inversely correlated to the tHcy level also in subjects with intact renal function (Wollesen et al, unpublished results). The physiological decline in renal function may partly explain the age effect (Wu et al 1994; Brattström et al 1994).

Diet and lifestyle influence the tHcy concentration. The intake of vitamin B_6, B_{12} and folate in food or supplements is inversely correlated to plasma tHcy (Selhub et al 1993). Smoking and coffee consumption cause a shift of the distribution towards higher tHcy

values, whereas physical activity is associated with low tHcy concentrations (Nygård et al 1995, 1997).

At present there are only limited data on tHcy in various ethnic populations. Ubbink and colleagues have shown that black South Africans have significantly lower tHcy concentrations than white South Africans (Vermaak et al 1991; Ubbink et al 1995b). In addition, different laboratories have different normal ranges for tHcy level (Ueland et al 1993). This may be partly explained by the use of different methodologies, but may also be related true ecogenetic differences, especially in relation to vitamin intake and the prevalence of the C677T mutation in the methylenetetrahydrofolate reductase gene (Motulsky 1996).

In an adult population, the tHcy distribution is skewed towards higher values. Notably, vitamin therapy makes the distribution curve more normal (Ubbink et al 1995a; Rasmussen et al 1996). In the Hordaland Homocysteine Study, we observed that subjects who do not smoke, drink little coffee and frequently take vitamin supplements have an almost normal tHcy distribution (Nygård et al, unpublished results). Children also seem to have a near normal tHcy distribution which becomes skewed in puberty (Ubbink et al 1996a; Tonstad et al, unpublished results). It is presently debated whether the normal tHcy range should be based on the distribution found in a healthy vitamin-supplemented group or that found in the general population (Rasmussen et al 1996).

CAUSES OF HYPERHOMOCYSTEINAEMIA

Hyperhomocysteinaemia is related to genetic or acquired conditions, or a combination of both (Motulsky 1996). Genetic diseases include the various forms of homocysteinuria. Of these, homozygosity for cystathionine β-synthase deficiency is the most common (Mudd et al 1995). Rare forms are severe defects of methylenetetrahydrofolate reductase, and low methionine synthase activity due to inborn errors of cobalamin metabolism (Rosenblatt and Cooper 1990).

Heterozygosity for cystathionine β-synthase deficiency is present in $<1-2\%$ of the general population (Mudd et al 1995), but these subjects have normal fasting tHcy concentration, although post-load tHcy may be elevated. Notably, recent enzymic and molecular genetic studies found no heterozygotes for cystathionine β-synthase among vascular patients with moderate hyperhomocysteinaemia (Engbersen et al 1995; Kozich et al 1995; Kluijtmans et al 1996).

A C677T mutation of methylenetetrahydrofolate reductase, characterized by reduced enzyme activity and thermolability, occurs in about 10% of the Caucasian population, and these subjects have a tendency towards moderate and intermediate hyperhomocysteinaemia (Kang et al 1991; Frosst et al 1995; Guttormsen et al 1996a). This C677T mutation may cause a redistribution of the folates (van der Put et al 1995), and the homozygous subjects probably need more folate to keep their tHcy concentrations within acceptable ranges. Recently, we observed that the majority of subjects with persistent moderate to intermediate hyperhomocysteinaemia had the C677T mutation in one or both alleles, but that a low daily dose (0.2 mg) of folic acid supplementation reduced the tHcy concentration in most subjects (Guttormsen et al 1996a).

Cloning of the human methionine synthase gene might lead to identification of other common genetic defects associated with hyperhomocysteinaemia.

Among the acquired conditions causing elevated tHcy, folate or cobalamin deficiency (Kang et al 1987; Allen et al 1994; Guttormsen et al 1996a) are most common. Both these vitamin deficiencies are associated with moderate and intermediate hyperhomocysteinaemia, and rarely, severe hyperhomocysteinaemia (Allen et al 1994). The relation between tHcy and blood/serum concentrations of folate and cobalamin is the basis for using tHcy as a marker of vitamin function. Although a sensitive parameter, hyperhomocysteinaemia is not specific for any vitamin deficiency. Determination of tHcy combined with serum creatinine, the blood vitamin concentrations, and/or methylmalonic acid (a specific marker of vitamin B_{12} function) will usually differentiate between the most common causes of hyperhomocysteinaemia.

Elevation of tHcy is also observed in disease states such as renal failure (Wilcken et al 1981; Dennis and Robinson 1996), acute leukaemia, psoriasis and hypothyroidism (Ueland et al 1992), and is induced by some drugs, i.e. methotrexate, nitrous oxide, antiepileptic agents, colestipol plus niacin, and agents acting as vitamin B_6 antagonists (Blankenhorn et al 1991; Refsum and Ueland 1990).

ASSESSMENT OF HOMOCYSTEINE STATUS

On the basis of the various factors reviewed in this article, we can list five points that the scientist or clinician should have in mind in the assessment of Hcy status.

(1) Are the sample collection procedures and Hcy method appropriate for the given purpose?
(2) Should methionine loading be included in the assessment?
(3) Should complementary blood analyses be performed? These include serum creatinine (renal function), serum vitamin B_{12} and folate, methylmalonic acid (vitamin B_{12} marker) and pyridoxal phosphate, and the C677T mutation.
(4) Can age, sex, medical conditions, drug use, diet, vitamin intake and lifestyle factors influence or explain the tHcy concentration of the subject?
(5) Does the tHcy concentration respond to vitamin therapy, in particular folic acid, vitamin B_{12} or vitamin B_6? A metabolic response may serve to identify the deficient vitamin.

CONCLUSION

Plasma tHcy determination is to an increasing extent used for diagnostic purposes and in scientific investigations. However, both the clinician and the scientist need to be aware of the multiple factors that may influence the plasma tHcy concentration. Appropriate precautions must be taken during blood sampling and processing to avoid an artificial increase in tHcy, especially when tHcy is used for cardiovascular risk assessment. The various assays for analysis of tHcy generally show good interassay agreement, but the methodology and performance differ, and reliable results usually depend on experienced personnel. Moreover, the assessment of the tHcy status frequently requires additional information about the subjects and the results of other biochemical analyses. Thus, the

tHcy concentration, although a valuable supplement to established diagnostic assays, should be interpreted with caution and not without detailed knowledge about both the patient and the laboratory management of the blood sample.

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