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Clinical implications of plasma homocysteine measurement in cardiovascular disease

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A link between plasma homocysteine concentrations and vascular disease was first suggested by the observation that patients with homocystinuria have premature thromboembolic disease. In patients with this inborn error of metabolism the concentration of homocysteine in plasma is profoundly raised (usually 10× normal). The recent upsurge in interest in this topic has been generated by the evidence that people with homocysteine concentrations in the upper part of the range for "normal" have an increased cardiovascular disease risk. It has been estimated that mild hyperhomocysteinaemia may be of similar significance to hypercholesterolaemia as a risk factor for premature atherosclerosis.[1] This article focuses on the place of homocysteine measurement as a risk factor for atherosclerotic vascular disease with less detail on thrombophilia, and only a brief mention of its place in assessing B12/folate status and monitoring homocystinuria.

Metabolism of homocysteine

Homocysteine is a sulphur containing amino acid derived from dietary methionine by demethylation (figs 1 and 2). Homocysteine may be further metabolised by the transulphuration pathway to cysteine, or remethylated using either methyltetrahydrofolate or betaine, which is confined to the liver. Vitamin B12 is a cofactor for methionine synthase and vitamin B6 (pyridoxal phosphate) is a cofactor for cystathionine β synthase and cystathionase. Raised concentrations of homocysteine may be caused by inherited enzyme defects or acquired deficiencies of vitamins B6, B12 or folate.

A deficiency of cystathionine β synthase is the usual enzyme defect in patients with classic homocystinuria (incidence approximately 1:200 000 population). This is inherited as an autosomal recessive trait. Heterozygosity probably does not carry additional cardiovascular disease risk. An inherited factor that may be of more general importance is a variant form of the enzyme methylene tetrahydrofolate reductase. This can lead to a reduced supply of methyltetrahydrofolate for remethylation of homocysteine. One mutation caused by a single base pair substitution (C677T) is remarkably common, having been found in its homozygous form in about approximately 10% of those populations tested.[2] This codes for an enzyme with slightly reduced activity. Subjects with this variant usually have no clinical consequences but are susceptible to development of raised homocysteine concentration in the presence of suboptimal folate intake. In a different context homozygosity for the C677T mutation, either in parent or child, appears to confer an increased risk of developing a neural tube defect in utero.[3]

Homocysteine as a risk factor for premature atherosclerosis

Hyperhomocysteinaemia in the range 15–30 μmol/l has been identified as a risk factor for premature atherosclerotic disease in numerous case-control studies including several large prospective studies.[1] This enhanced risk applies to coronary, cerebral, and peripheral vascular disease. It is unclear whether there is a threshold effect or whether there is a continuous effect across the range of homocysteine concentrations but with a steeply increasing effect at higher concentrations. Homocysteine potentiates other conventional risk factors such as hypertension and hyperlipidaemia and should not be interpreted in isolation.[4] As hyperhomocysteinaemia may be more important in the presence of these risk factors it is not logical to confine its measurement to individuals who have vascular disease but who lack conventional risk factors.

The mechanism by which homocysteine may cause vascular disease remains poorly defined. There is considerable evidence that homocysteine is toxic to vascular endothelial cells but the detailed processes involved require further elucidation.[5]

Although there is strong evidence linking homocysteine with cardiovascular disease, it remains to be proved that the relation is one of direct cause and effect. It is possible that homocysteine may be a marker for another factor (such as nutritional status). The results of intervention trials in which homocysteine concentrations are lowered by folic acid treatment are awaited to help resolve this point. Until such trials are completed it is premature

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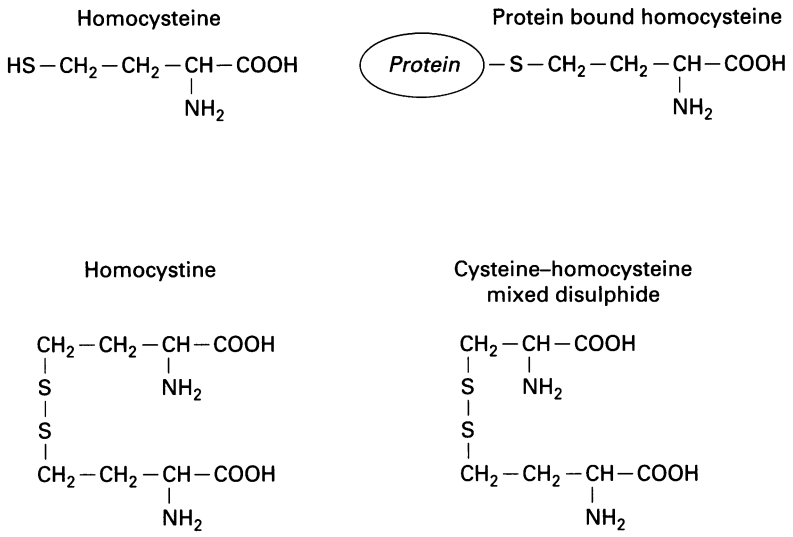

Figure 1 Forms of homocysteine in plasma.

to advise homocysteine measurement in all patients being assessed for cardiovascular disease risk. However, it is reasonable to include it in the profile for specialist centres and it should be measured in research studies, particularly if these include a nutritional component.

Thrombophilia

Patients with homocystinuria suffer from venous thromboembolism as well as arterial disease. This also applies to subjects with lesser degrees of hyperhomocysteinaemia, although the epidemiological evidence is much less extensive.[6] Disturbances in circulating coagulation factors have been described but reports are inconsistent and it is possible that all the vascular effects of homocysteine share the common mechanism of endothelial cell dysfunction.[7] In the absence of large intervention trials, the role of homocysteine measurements in thrombophilia is likely to remain uncertain but will probably become part of the screen for patients referred to specialist centres.

Homocysteine and vitamin status

Homocysteine concentrations are raised in the presence of deficiency of B12, folate or B6. This may occasionally be of value in monitoring B12 deficiency particularly in conjunction with measurement of methylmalonic acid, which is specifically raised in B12 but not folate deficiency. Suboptimal folate nutrition (less than 400 μg/day) is a common cause for mild hyperhomocysteinaemia in the general population, and has been postulated as part of the link between low intake of fruit and vegetables and premature cardiovascular disease. It is also possible that homocysteine may be implicated in the cause of neural tube defects associated with suboptimal folate intake.[3] Irrespective of whether there is folate deficiency, supplemental folic acid is effective in lowering plasma homocysteine concentrations by up to 40%.

Measurement of plasma homocysteine

CIRCULATING FORMS OF HOMOCYSTEINE

Homocysteine exists in normal human plasma in several different forms (fig 1). Approximately 70% is bound to plasma proteins, mainly albumin, by a disulphide link. The remaining homocysteine combines with other thiols, including cysteine, resulting in homocysteine–cysteine mixed disulphide (the most abundant disulphide species), and homocysteine itself, to form the dimer homocystine. Only a small proportion (approximately 1%) normally circulates as the free thiol compound. The different forms of homocysteine appear to be in a state of flux exchanging readily between the different forms. Also the redox status of homocysteine is linked with that of other plasma thiols, notably cysteine and glutathione. It is not known which form of homocysteine is directly involved in

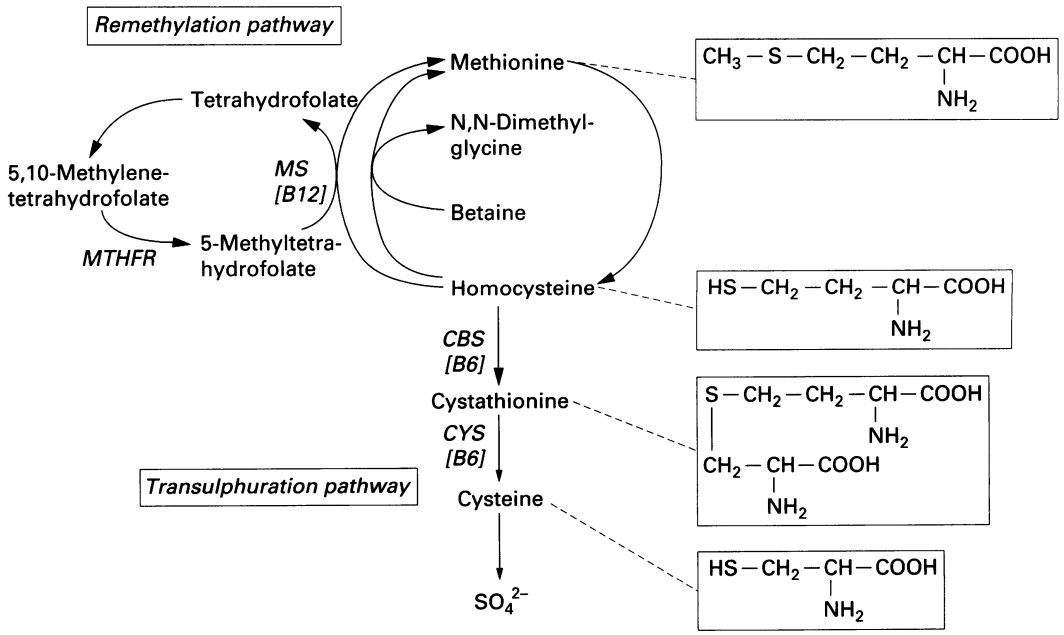

Figure 2 Metabolism of homocysteine. CBS, cystathionine β synthase; CYS, γ cystathionase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase.

pathological processes. Rapid oxidation and redistribution of homocysteine species takes place in freshly drawn plasma, which may greatly change the concentration of free reduced thiol but the concentration of total homocysteine is less labile. Specific determination of the individual fractions of homocysteine in plasma is difficult and is unsuitable for the laboratory in a routine clinical setting.[8] Hence measurement of homocysteine as a cardiovascular risk factor generally involves assay of total plasma homocysteine—that is, combination of bound, free, reduced, and oxidised forms. These assays involve the conversion of all forms into a single species by release from protein bound sites and reduction to the free thiol, which assumes that this represents biologically active homocysteine, either directly or indirectly. Several techniques can be used for the determination of total homocysteine in plasma.

Radioenzymatic assays

The principle of a radioenzymatic assay is the conversion of homocysteine to [^{14}C]S-adenosylhomocysteine, which is separated by chromatography (high performance liquid chromatography (HPLC), thin layer chromatography, or paper chromatography) and measured by scintillation counting.[9] Although specific, the laborious steps involved and precautions needed for the use of radioactive material make this unsuitable as a routine clinical method.

Immunoassay

A solid phase enzyme immunoassay has recently been developed by Axis Biochemicals (Oslo, Norway). Total homocysteine is enzymatically converted to S-adenosylhomocysteine, and this is measured by a competitive immunoassay with a horseradish peroxidase detection system in a microtitre plate format. This method appears to correlate well with HPLC methods and performs with a between-assay imprecision of 5–10%. The combination of an enzyme step and immunoassay for the product ensures specificity. This method may prove to be useful for large scale epidemiological type research but the enzyme incubation and microtitre format is not convenient for a rapid turnaround time in a clinical setting. However, a variation of this technique, which allows rapid assay of small numbers of samples, has been described for use on an Abbott IMx analyser, and this may soon be commercially available (Abbott, Maidenhead, UK).[10]

Ion exchange chromatography

A conventional amino acid analyser using postcolumn sample derivatisation with ninhydrin may be used for the determination of total or free homocysteine. Homocysteine can be eluted with a standard programme for physiological fluids,[11] but the main disadvantage of this method is the non-selectivity for sulphur amino acids, resulting in poor precision for homocysteine, long run times, and unnecessary expense. Amino acid analysis of methionine may be helpful in the monitoring of homocystinuria but is not needed for cardiovascular risk assessment.

Gas chromatography–mass spectrometry

Analysis of total homocysteine by gas chromatography–mass spectrometry in the selected ion monitoring mode involves sample pretreatment by addition of a deuterated internal standard and reducing agent, incubation, sample fractionation on a disposable anion exchange column, drying, and derivatisation with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide.[12] The method is specific, sensitive, and precise. The lack of total automation and the high cost of the equipment means that this method is not widely available or suitable for routine clinical use.

High performance liquid chromatography

HPLC with various methods of detection is currently the most widely used method for analysis of total plasma homocysteine. HPLC coupled to an electrochemical detector with a gold–mercury electrode has the advantage that a sample derivatisation step is not required. Problems such as contamination of the flow cell and deterioration of the gold–mercury electrode appear to have been overcome by the use of pulsed integrated amperometry.[13]

HPLC assays with fluorescence detection involve precolumn derivatisation with thiol specific fluorogenic reagents. The two best characterised methods involve the derivatising reagents monobromobimane (mBrB) or ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F).

mBrB couples with thiols at pH 8.0 to produce a highly fluorescent thioether. This occurs rapidly at room temperature and, in combination with appropriate sample handling equipment, allows the entire assay to be automated.[14] However, the reagent itself (mBrB), the hydrolysis products, and the impurities are fluorescent, and hence a gradient elution procedure is required for adequate separation and identification of homocysteine peaks.

SBD-F has a lower reactivity than mBrB, so that a longer incubation time (60 minutes) and high temperature (60°C) are required for its reaction with thiols. However, labelling of thiols by SBD-F is highly specific and no fluorescent degradation products are formed during the derivatisation procedure. This means that separation of SBD homocysteine from other SBD thiols can be achieved by isocratic (non-gradient) elution, producing clean chromatograms with straightforward identification and measurement. Also, the method lends itself to incorporation of an internal standard. SBD-F assays are currently the most widely used method for homocysteine assay in the external quality assessment scheme (ERNDIM see below). Our laboratory has adapted the method of Ubbink and colleagues[15] for determination of total plasma homocysteine and has incorporated cysteamine hydrochloride as an internal standard.[16]

Homocysteine assay by HPLC–SBD–F method

Plasma or homocysteine standard (120 µl) is combined with cysteamine (30 µl, 50 mol/l) and is incubated with 10% tri-n-butylphosphine in dimethylformamide (15 µl) for 30 minutes at 4°C to reduce and release protein bound thiols. Deproteinisation is achieved by addition of 10% trichloroacetic acid (150 µl) and centrifugation. An aliquot of the supernatant (50 µl) is mixed with sodium hydroxide (10 µl, 1.55 mol/l), borate buffer (125 µl, 0.125 mol/l, pH 9.5, containing 4 mmol/l EDTA) and SBD-F (50 µl) and incubated for 60 minutes at 60°C. The SBD-F derivative from the supernatant (20 µl aliquot) is eluted isocratically from the Spherisorb ODS2 column with mobile phase (KH_2PO_4, 0.1 mol/l, pH 2.0, containing 4% acetonitrile), at a flow rate of 0.8 ml/min. Cysteine, cysteamine, cysteinylglycine, homocysteine, and glutathione are detected by a fluorimetric detector (excitation 385 nm, emission 515 nm).

The assay is calibrated by reference to DL-homocysteine standards prepared by dissolving the pure compound in water. The use of other solvents (borate or phosphate buffer) is avoided because this can lead to under recovery of standards in the assay and hence spuriously high patient results.[16] Assay imprecision is 2.8% within batch and 6.9% between batch.

PREANALYTICAL FACTORS

Fasting

An increase in homocysteine would be expected postprandially because of the intake of methionine from protein. However, reported results show small and rather variable effects. In one study a protein rich meal (50 g protein) produced an increase in plasma homocysteine of 15–20%, which peaked six to eight hours after ingestion.[17] In contrast, a small but significant decrease in total plasma homocysteine was found two to four hours after consumption of a breakfast containing 25 g protein.[18] It has therefore been generally recommended that the subject should be fasting. However, in practice, samples taken in the non-fasting state are not likely to vary much from baseline providing that protein intake has been modest for the preceding 12 hours.

Methionine loading

Methionine loading can be used to stress the homocysteine metabolising pathways. The procedure involves oral intake of 0.1 g/kg methionine, and homocysteine is usually measured four or six hours after ingestion. It has been reported that the homocysteine concentration in a sample collected two hours after methionine loading is correlated with that of a four hour postmethionine sample, which may be a more convenient test in the clinical setting.[19] The original rationale behind the methionine loading test was to stress the transulphuration pathway and hence unmask partial enzyme deficiency caused by heterozygosity of cystathionine β synthase. However, as it is now evident that methionine loading will increase homocysteine irrespective of the metabolic defect, the need for this test should be questioned. Some studies have reported a greater discrimination between cases and controls[20] but in most the distinction is marginal. The uncertain reference values for postload results, the inconvenience for the investigator, and the unpleasant taste for the subject combine to make the case for abandoning methionine loading for routine clinical assessment and reserve it for studies designed to investigate homocysteine metabolic pathways.

Specimen collection

Total homocysteine in serum and plasma increases when separation from cells is delayed. The absolute increase in homocysteine in plasma is independent of the initial plasma concentration and may therefore cause larger proportionate errors in samples with normal plasma homocysteine content, whereas the relative increase is less likely to alter the clinical interpretation at higher basal concentrations (such as homocystinuria).[14] The rate of increase is approximately 0.5 µmol/l/hour during the first 24 hours at 22°C. If immediate centrifugation is not possible, the increase in homocysteine is reduced by keeping whole blood on ice until separation (maximum two hours). Once plasma has been separated from cells, total homocysteine concentration is relatively stable. Plasma samples can be kept at room temperature for up to 96 hours and can be posted without need for freezing. Long term storage is satisfactory at −20°C for at least six months.

Plasma rather than serum is recommended to facilitate rapid centrifugation. The most widely used anticoagulant is EDTA but others (heparin, citrate) appear to be acceptable. The use of tubes containing fluoride and oxalate, as used for glucose estimations, have been reported to inhibit the release of homocysteine from cells and hence reduce the need for rapid centrifugation[21]; therefore, there is a case for using this routinely. It has been suggested that fluoride and oxalate may interfere with enzyme immunoassays but this does not appear to be a problem with the widely used HPLC methods. A larger study with this specimen preservative to confirm reference ranges would allow this anticoagulant to be fully evaluated for clinical use.

INTERPRETATION

Compared with cholesterol measurement, standardisation of homocysteine assays is at a rudimentary stage. Analytical imprecision for HPLC assays, which are the most widely used, is usually in the range of 5–10% (between batch). For healthy individuals the between day biological variation is relatively small (coefficient of variation 7%) when repeated for periods up to one month, suggesting that a single measurement will characterise an individual's homocysteine concentration reasonably well.[22] With regard to accuracy, this will depend on the standard used for calibration. Most assays use solutions of DL-homocysteine for calibration. However, homocysteine is relatively unstable

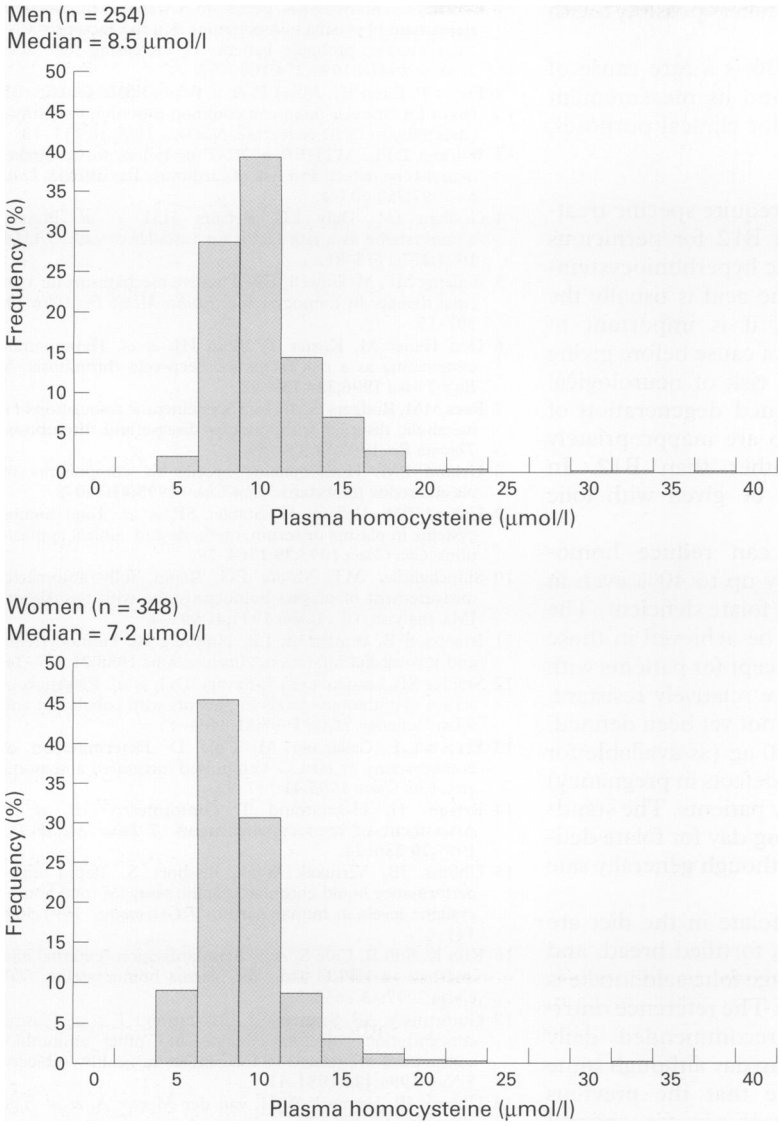

Figure 3 Distribution of plasma homocysteine concentrations in healthy adult blood donors.

and its purity is variable so this has led to the proposal that homocystine (oxidised dimer), which is reduced to free homocysteine in the assay, may be a more reliable standard.[23]

At present the only readily available external quality assessment scheme for the United Kingdom is the ERNDIM scheme (European Research Network for evaluation and improvement of screening diagnosis and treatment of Inherited Disorders of Metabolism). This scheme was originally set up to evaluate assays used for monitoring higher concentrations as found with inborn errors and hence the concentrations used are not particularly relevant to the use of the assay for cardiovascular risk assessment. Nevertheless, this scheme has assisted in a degree of between laboratory standardisation by highlighting variation. If homocysteine is to become fully established as a cardiovascular risk factor there is a need for a major initiative on assay standardisation. Despite these reservations, most HPLC assays give results with broadly similar distributions and reference ranges.[24] Figure 3 shows the data from the assay in our laboratory. The frequency distribution is slightly skewed towards higher values. Men have values approximately 1 μmol/l higher than women and values tend to increase slightly with age (data not shown). Data on total homocysteine in children are limited but do not appear to be very different from those in adults. Data on the effect of the acute phase response are limited. One study reported that homocysteine concentrations are lower two days after a stroke compared to those measured over one year later, but the numbers of subjects were small and these observations need to be confirmed and extended to other diseases.[25]

The effect of food and methionine loading have been discussed. Our current practice is to evaluate homocysteine status on a single sample taken from a patient who has not had a large protein meal within the preceding 12 hours. Values greater than 16 μmol/l are classified as raised; those greater than 30 μmol/l are considered markedly raised; and those greater than 100 μmol/l are likely to indicate homocystinuria or another metabolic disorder.

Further investigation of moderate hyperhomocysteinaemia

Subjects with homocysteine concentrations above 16 μmol/l warrant further investigation. The most common cause in hospital patients is renal impairment. Individuals with creatinine concentrations in the range 150–500 μmol/l typically have homocysteine values in the range 20–30 μmol/l. The mechanism for this is unclear although it does appear to be related to impaired metabolism of homocysteine by the kidney rather than impaired filtration into the urine, which is a very minor route for direct homocysteine clearance.[20] It has been proposed that hyperhomocysteinaemia in renal failure may explain partly the accelerated atherosclerosis that is a feature of the condition. Hyperhomocysteinaemia in renal failure is relatively resistant to treatment with vitamins, although some reduction may be achieved with folic acid.

Folate and B12 status should also be assessed. Patients with frank vitamin deficiency will generally have homocysteine values greater than 20 μmol/l (for example, pernicious anaemia) but borderline vitamin status alone gives rise to borderline homocysteine values. If available, the measurement of methylmalonic acid may be helpful, being raised in B12 but not folate deficiency. In equivocal cases it may be reasonable to treat with a vitamin supplement and monitor the homocysteine response.

Where available, determination of methylene tetrahydrofolate reductase genotype may help refine the diagnosis. This can be determined by relatively simple techniques based on the polymerase chain reaction.[2] Most individuals who are homozygous for this mutation have "normal" homocysteine concentrations, but in the presence of suboptimal folate intake may develop hyperhomocysteinaemia.[26] Heterozygosity for the mutation is likely to have no clinical significance. The demonstration of homozygosity for methylene tetrahydrofolate reductase variant in conjunction with raised homocysteine would generally be an indication

for enhancing folate intake, possibly with supplements.

Deficiency of vitamin B6 is a rare cause of hyperhomocysteinaemia and its measurement is not generally required for clinical purposes.

Treatment

Specific disorders clearly require specific treatment (such as parenteral B12 for pernicious anaemia). For non-specific hyperhomocysteinaemia, treatment with folic acid is usually the most effective. However, it is important to exclude B12 deficiency as a cause before giving folic acid because of the risk of neurological damage (subacute combined degeneration of the cord) in patients who are inappropriately prescribed folic acid rather than B12. In equivocal cases B12 can be given with folic acid.

Folic acid treatment can reduce homocysteine concentrations by up to 40% even in those who are not frankly folate deficient. The greatest effect appears to be achieved in those with the highest values, except for patients with renal impairment, who are relatively resistant.

The optimum dose has not yet been defined. A daily dose as low as 400 μg (as available for prevention of neural tube defects in pregnancy) may be sufficient for many patients. The standard prescribed dose of 5 mg/day for folate deficiency is supramaximal although generally safe (see caution above).

The major sources of folate in the diet are fortified breakfast cereals, fortified bread, and fruit and vegetables. Average folic acid intake is approximately 280 μg/day. The reference nutrient intake (previously recommended daily allowance (RDA)) is 200 μg/day although some nutritionists would argue that the previous RDA of 400 μg/day is required for optimal nutrition.

Addition of B6 supplements has been generally reported to have little additional homocysteine lowering effect but can be added in doses of 10–25 mg/day.[27]

Summary

Plasma homocysteine is emerging as an important risk factor for cardiovascular disease. Measurement in clinical laboratories is feasible by several techniques, including HPLC. Of particular importance is the potential for treatment by dietary modification and vitamin supplements, particularly folic acid. The outcome of vitamin intervention on cardiovascular events is awaited with interest. If the role of homocysteine is confirmed then it may rival cholesterol in importance as a cardiovascular risk factor.

Appendix

Secretary of ERNDIM is Dr Brian Fowler, Basler Kinderspital, Roemergasse 8 VH 4005 Basel, Switzerland (fax:+ 41 61 692 6555).

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