Assessment of homocysteine as a cardiovascular risk factor in clinical practice

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SUMMARY. Elevated plasma total homocysteine concentrations are a marker of vitamin deficiency and a risk factor for cardiovascular disease. It is possible that vitamin supplementation with folic acid and other B vitamins, which lower plasma homocysteine concentrations, may reduce the risk of cardiovascular disease. Large-scale clinical trials are currently underway to assess the homocysteine hypothesis of cardiovascular disease. Pending the outcome of such trials, measurement of plasma homocysteine concentrations in people at high risk of cardiovascular disease may help to identify patients who could benefit from more intensive treatment of classical cardiovascular risk factors. The introduction of immunoassays for homocysteine determination has made assessment of homocysteine status accessible to most routine hospital laboratories, and this review summarizes the evidence on why and how to assess homocysteine as a risk factor for cardiovascular disease in clinical practice.

Despite a reduction in mortality from cardiovascular diseases over the last few decades, the mortality caused by these diseases in the UK is among the highest in the world. Cardiovascular diseases constitute the single most common cause of mortality in most Western populations.[1] Elevated blood cholesterol concentrations, high blood pressure and cigarette smoking explain much of the differences in cardiovascular disease rates within and between populations.[2] In addition to the established importance of lowering blood pressure, recent clinical trials have clearly demonstrated that lowering blood cholesterol concentration is associated with highly significant reductions in coronary heart disease (CHD), stroke and 'all-cause' mortality.[3–5]

Research interest has expanded into the study of emerging risk factors for cardiovascular disease, including homocysteine. Over the last decade, evidence has accumulated that elevated plasma homocysteine concentrations are associated with an increased risk of atherosclerotic and thrombo-embolic events.[6–8] Plasma concentrations of homocysteine reflect genetic and environmental factors, including diet.[9,10] Vitamin supplementation with folic acid and vitamin B_{12} achieves substantial reductions in blood homocysteine concentrations.[11] Several large-scale trials are currently underway to assess whether vitamin supplementation to lower homocysteine concentrations can reduce the risk of vascular disease.[12] This review describes the metabolism of homocysteine, including its vitamin dependence, genetic and environmental determinants of homocysteine, the relevance of homocysteine as a risk factor for cardiovascular disease and practical issues in the assessment of homocysteine status.

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METABOLISM

Homocysteine is a sulphur amino acid derived from methionine, following the loss of a methyl group. The formulae of these and related metabolites are shown in Fig. 1. Homocysteine lies at a branch point in one-carbon metabolism between two metabolic pathways (remethylation and trans-sulphuration) in all cells (*see* Fig. 2). In the remethylation pathway, homocysteine accepts a methyl group from the methyl-tetrahydrofolate to form methionine. The remethylation

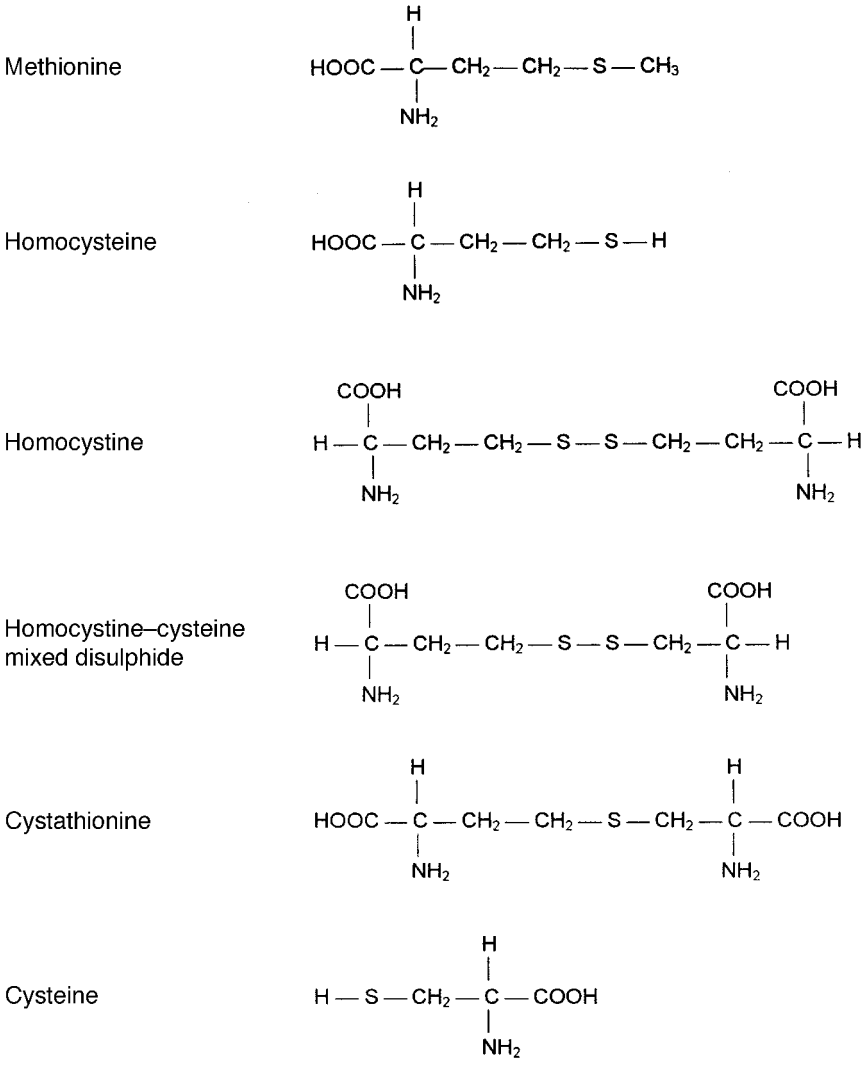

FIGURE 1. *Formulae of methionine and homocysteine and related metabolites.*

reaction, catalysed by methionine synthase (MS), requires vitamin B_{12} as a cofactor and methyltetrahydrofolate as substrate. Methylene-tetrahydrofolate reductase (MTHFR) plays an important role in remethylation, by supplying methyl groups as methyl-tetrahydrofolate for homocysteine remethylation. An alternative remethylation pathway utilizing betaine as methyl donor is confined to the liver. Much of the methionine is activated to form S-adenosylmethionine (SAM), which is the chief donor of methyl groups for methyl transferases involved in the synthesis of DNA, proteins and phospholipids. The loss of the methyl group from SAM results in the production of S-adenosylhomocysteine (SAH), which is in turn hydrolysed to form homocysteine.

In the trans-sulphuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalysed by vitamin B_6-dependent cystathionine-β-synthase (CS). Under normal circumstances the flow of methionine to cystathionine accounts for half of all methionine metabolism. The remaining half of total body homocysteine is remethylated by methyl groups derived in equal amounts from methyltetrahydrofolate or betaine.[13] The intracellular levels of homocysteine

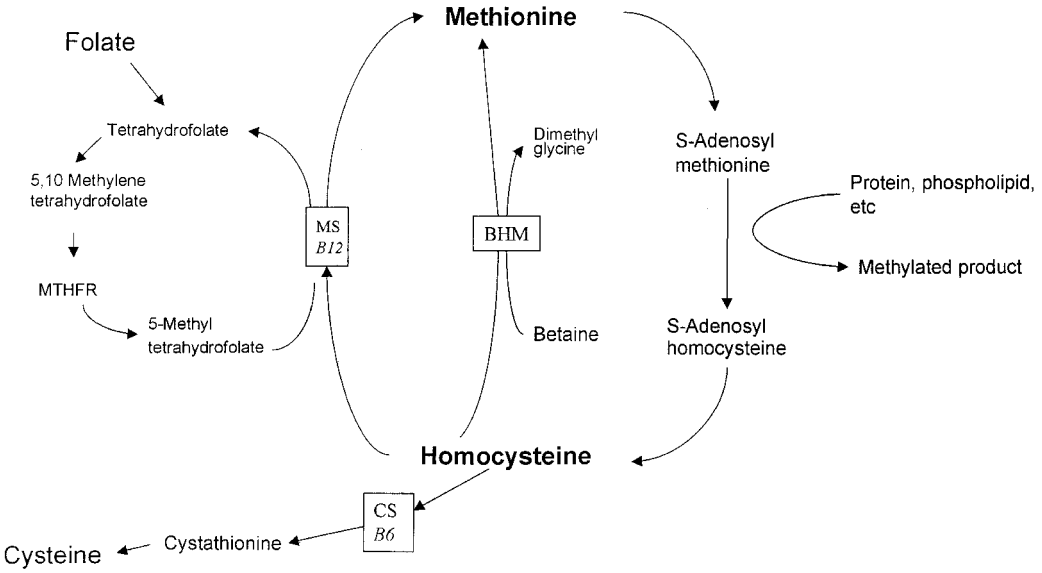

FIGURE 2. *Summary of one-carbon metabolic pathways. BHM = betaine–homocysteine methyltransferase; CS = cystathionine-β-synthase; MS = methionine synthase; MS B12 = B_{12}-dependent MS; CS B6 = B_6-dependent CS; MTHFR = 5,10-methylenetetrahydrofolate reductase; SAM = S-adenosylmethionine; THF = tetrahydrofolate.*

are highly regulated, and any increased production is met by export from cells. Thus, blood levels of homocysteine reflect intracellular concentrations of homocysteine and the homeostatic balance of the enzymes involved in methionine metabolism to ensure a supply of methyl groups for essential reactions in all cells.

GENETIC INFLUENCES

Homocystinuria and severe hyperhomocysteinaemia (> 100 μmol/L) are usually caused by rare inborn errors of metabolism.[14] Cystathionine-β-synthase deficiency is the most common genetic cause of severe hyperhomocysteinaemia, with an estimated frequency of 1 per 300 000 live births. Approximately 1% of the general population is heterozygous for CS deficiency and these have homocysteine levels in the range of 20–40 μmol/L.[15] Heterozygotes for CS deficiency have higher risks of occlusive vascular disease.[14] A thermolabile variant of MTHFR due to a C→T substitution in the gene (C677T) encoding this enzyme is a much more frequent mutation, affecting 5–15% of the population.[16] The C677T mutation for MTHFR causes a single amino acid substitution which results in altered binding of folate.[16] Individuals who have the TT mutation have about 25% higher homocysteine levels than do individuals with the CC variant, depending on their folate status. Individuals with this mutation are particularly sensitive to folate and riboflavin deficiency.[17,18]

Despite the association of MTHFR mutation with elevated homocysteine levels, the evidence relating this mutation with cardiovascular disease is conflicting.[19,20] Some of the discrepant results between individual studies may relate to differences in dietary intake of folate and riboflavin levels in these populations, whereby the effects of this mutation on cardiovascular risk are chiefly manifest in the presence of folate and riboflavin deficiency.[17,18] Other recently discovered genetic variants include defects of methionine synthase[21] and methionine synthase reductase,[22] but these have less significant effects on homocysteine levels than MTHFR.

DIETARY INFLUENCES

Dietary intake of folate, vitamin B_{12} and vitamin B_6 are the chief nutritional determinants of blood homocysteine concentrations, with folate being the predominant vitamin.[9,10] Observational studies show a linear relationship of increasing blood folate levels and a decreasing homocysteine concentration with increasing intake of dietary folate. The Framingham study

demonstrated that those in the top decile of dietary folate intake had homocysteine levels that were about 5 μmol/L lower than did those in the bottom decile.[9] More reliable evidence for the effects of individual vitamins on blood homocysteine levels comes from the clinical trials comparing the effects of such vitamins on homocysteine levels.

Combined analysis of the effects of these vitamin trials suggest that folic acid in a dose ranging from 0·5 to 5 mg daily was associated with reductions in homocysteine levels of about 25%.[11] The addition of vitamin B_{12} to folic acid was associated with further reductions in homocysteine levels of about 7%. Hence, a multivitamin combination of folic acid and vitamin B_{12} was associated with reductions in homocysteine levels of about one-third (i.e. from a mean concentration of 12 to 8 μmol/L). Vitamin B_6 supplementation did not appear to affect basal homocysteine levels, but was associated with a reduction in homocysteine levels after methionine loading.[11]

ASSESSMENT OF HOMOCYSTEINE STATUS

In plasma, 70% of homocysteine is bound to albumin, the remaining 30% forming disulphides with other thiols, and only 1% circulates in a free form. The sum of these homocysteine forms is referred to as total homocysteine or homocyst(e)ine. Assays of 'total homocysteine'[23,24] convert these multiple unstable thiols to a reduced form which can be measured directly or, after derivatization, by high-performance liquid chromatography [HPLC; coefficient of variation (CV) $\geqslant$ 5%] or gas chromatography–mass spectrometry (GCMS; CV 4%).

The introduction of immunoassays for homocysteine determination now affords a highly practical and cost-effective method for homocysteine determination.[25–27] AXIS-Shield (Oslo, Norway) has developed two variants of immunoassay for homocysteine determination, one a fluorescence polarization immunoassay (FPIA) which runs on the IMx system (Abbott Laboratories, Chicago, USA), and the other an enzyme immunoassay (EIA) that can be carried out on a microtitre plate.[27] The intra-laboratory imprecision is less than 5% for the FPIA method and less than 9% for the EIA method using automated pipetting.[27] Figure 3 shows a comparison of the performance of the FPIA and EIA immunoassays with those carried out by GCMS as part of a multicentre European Demonstration project.[27] The Demonstration project showed that the immunoassays for homocysteine determination could safely be used in place of assays carried out by HPLC or GCMS. A comprehensive review of the performance of the different homocysteine assays, including sample volume, analytical imprecision, throughput and quality control schemes, has recently been published.[28]

Meticulous attention to detail in sample handling is important for accurate homocysteine determination. Plasma concentrations of homocysteine rise rapidly unless plasma is separated from red cells immediately or kept chilled at 4°C. Homocysteine levels in unseparated whole blood stored at room temperature (in the UK approximately 23°C) increase by about 10% per hour, and higher ambient temperatures result in a greater increase. After prompt separation of red cells, homocysteine concentrations in plasma remain stable for several days at room temperature or 4°C and for several years at $\div$20°C. Plasma samples can therefore be sent to laboratories by first-class post for analysis. EDTA is a convenient anticoagulent (as is lithium heparin); serum is less desirable because the inevitable separation delay leads to elevations in homocysteine concentration. An alternative to prompt separation is the stabilization of blood homocysteine by 3-deaza-adenosine[29] or fluoride (the amount of fluoride in a fluoride–oxalate tube is quite adequate).

Plasma homocysteine concentration is higher in men than in women and increases with age, renal impairment and following a meal containing a significant amount of methionine and certain drugs (*see* Table 1). The mean homocysteine concentration also varies according to an individual's dietary intake of folate and other vitamins. Assessment of homocysteine status therefore requires age- and sex-related reference ranges. But for most practical purposes, the normal middle-aged adult plasma concentrations of total homocysteine lie between 8 and 15 μmol/L. For individuals aged 65 years or greater, a plasma homocysteine concentration of 20 μmol/L or greater would be considered abnormal. The American Heart Association have arbitrarily defined hyperhomocysteinaemia as being divided into moderate, intermediate and severe, referring to concentrations $\geqslant$15–30, 30–100 and $\leqslant$100 μmol/L, respectively.[30] Homocysteine concentration within individuals remains relatively stable at least for a year,[31] and the

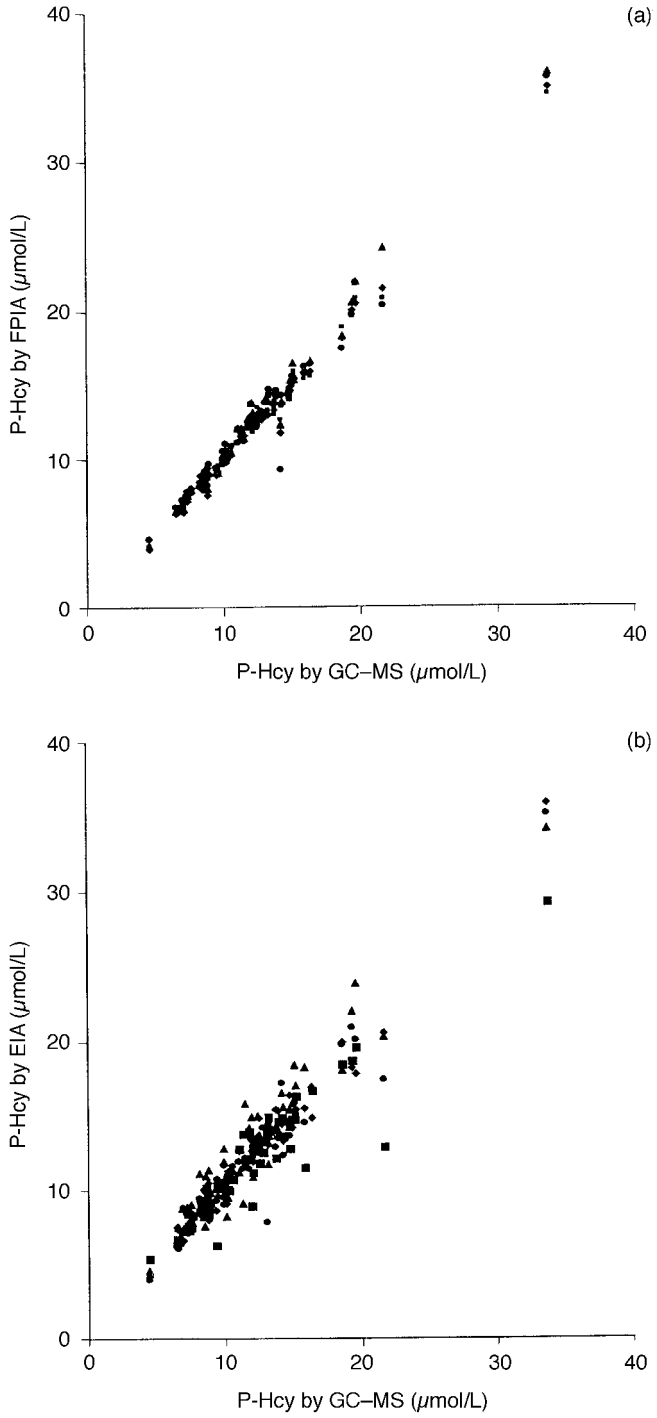

FIGURE 3. *Homocysteine concentrations (P-Hcy) in 57 patient samples by gas chromatography–mass spectrometry (GC–MS) compared with two variants of immunoassay, (a) a fluorescence polarization immunoassay (FPIA) which runs on the IMx system, and (b) an enzyme immunoassay (EIA) that can be carried out on a microtitre plate. Data from reference 27. A different symbol is used for each of the four participating laboratories.*

TABLE 1. *Causes of hyperhomocysteinaemia*

Increased age
Male gender
Lack of exercise
Smoking
Kidney disease
Organ transplantation
Hypothyroidism
Psoriasis
Vitamin deficiencies (folate, vitamin B_{12}, vitamin B_6, riboflavin)
Enzyme deficiencies (cystathionine synthase, methionine synthase, MTHFR C677T variant)
Drugs (corticosteroids, ciclosporin, methotrexate, phenytoin, theophylline, fibrates)

MTHFR = methylenetetrahydrofolate reductase.

within-person standard deviation in homocysteine concentrations is 1 μmol/L within one year.[10]

A single blood sample, which need not be from a fasting patient, is the most widely used investigation to assess homocysteine status, and in practice a light breakfast has little influence on it.[7] Oral methionine loading (0·1 g/kg of L-methionine) leads to about a three-fold increase in plasma homocysteine concentration at 6 h and detects latent disorders of remethylation and trans-sulphuration. It was originally used to identify carriers for homocystinuria, but more recently has been used to identify latent hyperhomocysteinaemia regardless of the aetiology. While it has been suggested that some people with a normal fasting plasma homocysteine may have hyperhomocysteinaemia revealed by the methionine-loading test, the relevance of this to clinical outcomes is uncertain.[32–35] The actual cut-off points for an abnormal methionine loading test have been defined as being $\leqslant 2$ standard deviations (SD) above the mean level in controls (i.e. $\leqslant$35–40 μmol/L, depending on age). Folate concentration and MTHFR TT genotype influence the post-methionine increase in homocysteine, as does gender,[33] women showing greater increases than men. However, homocysteine concentration after methionine loading is highly correlated with random basal homocysteine concentration, measurement of which is easier to perform, cheaper and less unpleasant for the patient. Also, there is substantial uncertainty about the reproducibility of homocysteine concentration measurement in response to methionine loading, which may explain the conflicting results about the predictive value of methionine load-induced homocysteine concentration as a risk factor for cardiovascular disease, independent of random or 'basal' homocysteine concentration. Hence, methionine loading tests should probably be restricted to research purposes.

EVIDENCE RELATING HYPERHOMOCYSTEINAEMIA TO VASCULAR DISEASE

There is now evidence from a large number of epidemiological studies conducted in different settings of a positive association between blood concentration of homocysteine and risk of cardiovascular disease.[6–8] Thus, patients with heart disease,[36–42] stroke,[43,44] peripheral vascular disease[45–47] and thromboembolic disease have higher blood concentrations of homocysteine than do age-matched individuals who are disease-free. Throughout the typical range of plasma homocysteine concentrations encountered in Western countries (8–15 μmol/L), lower concentrations are associated with lower risks of vascular disease. As stated above, plasma concentrations of homocysteine increase with age and are greater in men than in women and greater in current smokers than in non-smokers.[7] Plasma concentrations of homocysteine are positively correlated with levels of systolic blood pressure, creatinine and with total and LDL-cholesterol, and inversely correlated with HDL-cholesterol and current alcohol consumption.[7] Despite this correlation of plasma homocysteine with other cardiovascular risk factors, the associations of homocysteine with vascular risk appear to be independent of other cardiovascular risk factors.[8]

Prospective studies show that differences in concentrations of plasma homocysteine precede the onset of disease, which would appear to argue against differences in homocysteine concentrations being simply a marker of sub-clinical disease.[36–42] An overview of published studies[8] shows that the odds ratio for coronary disease associated with a 5 μmol/L increase in homocysteine concentration in prospective studies was 1·3 [95% confidence interval (CI): 1·1 to 1·5], which is less marked than that observed in retrospective studies with population controls, in which the odds ratio was 1·6 (95% CI: 1·4 to 1·7), or in retrospective studies with volunteer controls, in which the odds ratio was 1·9 (95% CI: 1·6 to 2·3). Some of the discrepant results of

prospective studies may relate to differences in the mean time to events in such studies. There is a trend whereby the strength of association of plasma homocysteine concentration with cardiovascular disease appears to attenuate at longer intervals of follow-up in such studies. These issues are currently being addressed in a meta-analysis of individual patient data from all the available observational studies of homocysteine and cardiovascular disease.

EVIDENCE OF CLINICAL BENEFIT FROM VITAMIN SUPPLEMENTATION

Observational studies cannot exclude the influence of confounding factors, including dietary factors, which may explain the modest relative differences observed. The results of long term clinical trials of vitamin therapy to lower blood homocysteine concentrations, conducted in high-risk populations, are required to assess whether the observed associations are causal. Several large-scale trials are currently under way testing the effects of homocysteine-lowering therapy in high-risk patients. In the UK, the SEARCH (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine) trial is currently testing the effects of folic acid (2 mg) and vitamin B_{12} (1 mg) administered daily versus placebo in 12 000 patients who have had a previous myocardial infarction.[12] This trial is one of seven large-scale trials that will provide randomized evidence for the effects of homocysteine-lowering therapy in 40 000 patients with prior heart disease.[12] The VISP (Vitamin Intervention for Stroke Prevention) and VITATOPS (Vitamins to Prevent Stroke) trials will provide randomized evidence for the effects of homocysteine-lowering therapy in patients with prior stroke.[12] The VITRO (Vitamins and Thrombosis) trial will assess the effects of homocysteine-lowering therapy for thromboprophylaxis in patients with previous venous thrombosis and the FACIT (Folic Acid and Carotid Intima-media Thickness) trial will assess the effects of homocysteine lowering on carotid artery intima–media wall thickness in healthy elderly patients.

The results of these large-scale trials are required to establish whether the associations of homocysteine with vascular risk are causal or not. Consequently, definitive recommendations about widespread screening of patients for elevated homocysteine concentrations cannot be justified until it is clearly demonstrated that use of vitamin supplements to lower homocysteine concentration is associated with a reduction in cardiovascular risk. However, individual clinicians may wish to measure homocysteine concentration as a marker of risk in patients presenting with coronary heart disease at an early age or with a paucity of other known risk factors. Individuals with prior cardiovascular disease and moderately elevated homocysteine concentrations should have careful assessment and appropriate treatment of established risk factors such as smoking, elevated blood pressure and elevated blood cholesterol concentrations.

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