

The Summer Meeting of the Nutrition Society was held at the University of Ulster at Coleraine on 24–28 June 1996

Postgraduate Symposium

The effects of oral vitamin supplementation on cardiovascular risk factors

BY J. V. WOODSIDE^{1,2}, I. S. YOUNG², J. W. G. YARNELL¹, D. McMASTER³
AND A. E. EVANS¹

¹Department of Epidemiology and Public Health, ²Department of Clinical Biochemistry and ³Department of Medicine, Institute of Clinical Science, The Queen's University of Belfast, Belfast BT12 7AR

CHD causes approximately half the deaths among middle-aged adults in the industrialized world. However, major accepted risk factors combined can explain only about 50% of heart disease (Editorial, 1984). The possible aetiological involvement of novel risk factors, therefore, is receiving much attention.

The present review focuses on two such risk factors: hyperhomocysteinaemia and LDL oxidation. Both these factors can be linked with inadequate vitamin intake and, therefore, may be amenable to nutritional intervention (Selhub *et al.* 1993; Jha *et al.* 1995).

HYPERHOMOCYSTEINAEMIA

Homocysteine is a S-containing amino acid which is an intermediary product in methionine metabolism (Finkelstein, 1990). Recent investigations have focused on the possibility that moderate elevations may be associated with increased risk of vascular disease (McCully, 1983). To date, more than twenty clinical studies involving over 2000 patients with cardiovascular disease and a similar number of controls have shown that patients tend to have higher homocysteine levels, even though in most cases values are within the accepted normal range (Malinow, 1990; Kang *et al.* 1992; Ueland *et al.* 1992).

Several retrospective and cross-sectional studies have linked premature vascular disorders including CHD, cerebral and peripheral vascular disease with elevated homocysteine levels (Malinow, 1990; Clarke *et al.* 1991; Malinow *et al.* 1993; Boushey *et al.* 1995). In addition, the association between hyperhomocysteinaemia and cardiovascular disease has been confirmed in several large prospective studies (Taylor *et al.* 1991; Stampfer *et al.* 1992; Verhoef *et al.* 1994; Arnesen *et al.* 1995; Perry *et al.* 1995) with only one study showing no association (Alfthan *et al.* 1994). In the Physicians' Health Study, a total of 14 916 US male physicians aged 40 to 84 years were followed up for 6 years. Men with homocysteine levels above the 95th percentile (based on control distribution) had a three-fold increased risk of myocardial infarction compared with those in the bottom 90% (Stampfer *et al.* 1992). The findings were also statistically compatible with a graded risk increase across the distribution, a suggestion confirmed by Perry *et al.* (1995) in a prospective study of stroke in middle-aged British men. Similar findings have been reported for myocardial infarction (Arnesen *et al.* 1995), carotid-artery thickening (Malinow *et al.* 1993) and angiographically-defined coronary artery stenosis (Genest *et al.* 1990). In addition, Selhub *et al.* (1995) demonstrated a gradual increase in the prevalence

of carotid artery stenosis with increasing levels of homocysteine. Meta-analysis by Boushey *et al.* (1995) showed an increase in risk of coronary artery disease of about 70 % for each 5 $\mu\text{mol/l}$ rise in fasting homocysteine.

Several mechanisms are likely to be involved in the induction of vascular disease by homocysteine, including endothelial cell desquamation (Harker *et al.* 1974; Starkebaum & Harlan, 1986), oxidation of LDL (Heinecke *et al.* 1987; Parthasarathy, 1987; Blom *et al.* 1995), and monocyte adhesion to the vessel wall (Kottke-Marchant *et al.* 1990). Additional roles for homocysteine in haemostasis and atherogenesis have been suggested but not confirmed. Early studies showed that in hyperhomocysteinaemic patients, platelet turnover was increased (Harker *et al.* 1974), but this has not been reproduced (Uhlemann *et al.* 1976). Coagulation protein function may be disturbed as indicated by low factor VII levels (Munnich *et al.* 1983) and reduced functional anti-thrombin III activity (Giannini *et al.* 1975). It has been suggested that a hypercoagulable state is associated with hyperhomocysteinaemia since there have been many reports of altered endothelial cell function, including enhanced factor V activity (Rodgers & Kane, 1986), decreased protein C activation (Rodgers & Conn, 1990), diminished fibrinolysis (Harpel *et al.* 1992) and increased tissue factor activity (Rodgers *et al.* 1993). It must be noted, however, that these effects are not homocysteine-specific; a variety of free thiol-group amino acids show similar tendencies (Rees & Rodgers, 1993).

Metabolism

Homocysteine is formed as a product of the S-adenosyl-L-homocysteine hydrolase (*EC* 3.3.1.1) reaction which is responsible for the removal of S-adenosyl homocysteine, a product of S-adenosyl methionine-dependent transmethylation. Intracellular homocysteine is (1) remethylated to methionine, the transmethylation pathway; (2) converted to cystathionine, the trans-sulphuration pathway, or (3) exported from the cells (Fig. 1). Pathway 1 is catalysed by the enzyme methionine synthase (*EC* 2.1.1.13) which requires cobalamin (vitamin B₁₂) as a cofactor; the methyl donor is 5-methyltetrahydrofolate. During pathway 2, the vitamin B₆-dependent enzyme cystathionine β -synthase (*EC* 4.2.1.22) catalyses the irreversible condensation of homocysteine with serine to form

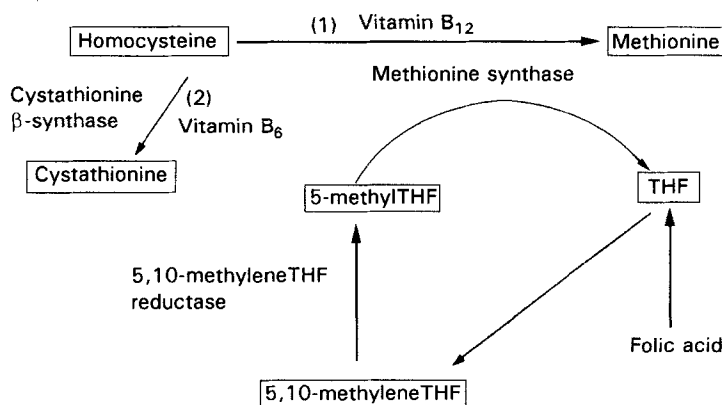


Fig. 1. Metabolism of homocysteine. THF, tetrahydrofolate; methionine synthase, *EC* 2.1.1.13; cystathionine β -synthase, *EC* 4.2.1.22; 5,10-methylene THF reductase, *EC* 1.7.99.5.

cystathionine. Release of homocysteine into the extracellular medium represents the third route of cellular homocysteine disposal.

Measurement of homocysteine

Homocysteine exists in several forms in plasma from healthy subjects with 70% of homocysteine from healthy subjects associated with plasma protein (Ueland *et al.* 1993). The sum of all homocysteine species in plasma (free plus protein-bound) is referred to as total homocysteine (tHcy). To measure total homocysteine, plasma is first subjected to a reducing agent to cleave the disulphide bonds of homocysteine, homocysteine mixed disulphide and protein-bound homocysteine. The plasma is then deproteinized and homocysteine levels measured. The range for total homocysteine values differs somewhat from one laboratory to another, but values between 5 and 15 $\mu\text{mol/l}$ are usually considered as normal (Ueland *et al.* 1993).

Homocysteine can be measured in plasma after fasting or after an oral methionine load. In the meta-analysis of Boushey *et al.* (1995), the summary risk estimates based on studies which measured fasting levels were of similar magnitude to those based on fasting and post-load; suggesting that both fasting and post-load homocysteine are equally strongly related to risk of cardiovascular disease.

Causes of hyperhomocysteinaemia

Total plasma homocysteine seems to be dependent on age, gender, and menopausal status (Ueland *et al.* 1993). Moreover, both environmental and genetic influences contribute to hyperhomocysteinaemia.

Environmental and dietary factors. Cross-sectional and experimental evidence suggests that mild hyperhomocysteinaemia may be related to sub-optimal levels of folic acid, pyridoxine and cyanocobalamin, all cofactors in homocysteine metabolism (Park & Linkswiler, 1970; Slavik *et al.* 1982; Smolin & Benevenga, 1982; Smolin *et al.* 1983; Kang *et al.* 1987; Brattstrom *et al.* 1988; Lindenbaum *et al.* 1988; Stabler *et al.* 1988; Miller *et al.* 1992). In a study of vitamin and homocysteine levels in an elderly population, Selhub *et al.* (1993) found a strong inverse correlation between homocysteine and plasma folate, and weaker inverse correlations between homocysteine and cobalamin and homocysteine and pyridoxal-5-phosphate. The authors concluded that elevated homocysteine levels could, in great part, be due to vitamin status. Ubbink *et al.* (1993) produced similar results and confirmed by intervention that a daily supplementation of folic acid, pyridoxine and cyanocobalamin could normalize elevated homocysteine concentrations within 6 weeks. Ubbink *et al.* (1994) then looked at the effect of supplementation with the individual vitamins over 6 weeks. Folic acid supplementation (0.65 mg/d) reduced plasma homocysteine concentrations by 41.7% and cyanocobalamin (0.4 mg/d) by 14.8%. The pyridoxine supplement (10 mg/d) had no significant effect on homocysteine concentrations. The combination of the three vitamins reduced circulating homocysteine concentrations by 49.8% which was not significantly different from the reduction achieved by folate supplementation alone. No trial has, as yet, measured the effectiveness of vitamin supplementation with a clinical endpoint, i.e. a reduction in cardiovascular events.

The question arises as to whether dietary change in B-vitamin intake can reduce plasma homocysteine, or whether food fortification or vitamin supplementation is necessary. Selhub *et al.* (1993) found that 400 μg folate/d was necessary to prevent elevation of homocysteine and this intake was only attained by 30–40% of participants in

the Framingham study. Boushey *et al.* (1995) assessed that if the US population were to eat two to three more servings of fruit and vegetables daily, this would lead to a reduction of 4 % in deaths from cardiovascular disease annually through homocysteine reduction.

Cuskelly *et al.* (1996) looked at the effects of increasing dietary folate on erythrocyte folate. Erythrocyte folate concentration increased significantly over 3 months in subjects taking folic acid supplements or food fortified with folic acid (such that folic acid intake increased by 400 µg/d). There was no increase in those given food naturally rich in folate (again to provide an estimated increase in intake of 400 µg/d) and those offered dietary advice only. Their likely explanation lies in the known higher bioavailability of folic acid compared with food folates. Further investigation is needed using larger numbers of subjects to assess the relative effectiveness of a high-folate diet, food fortification with folate and folic acid supplementation on plasma homocysteine.

Genetic factors. The classical syndrome homocystinuria defines a group of inherited disorders characterized by the excretion of large amounts of homocysteine in the urine. Common clinical signs are mental retardation, ectopic lens, premature vascular disease and thrombosis (Ueland *et al.* 1992; Mudd *et al.* 1989). The most common cause of homocystinuria is homozygosity for cystathionine β-synthase deficiency which has a prevalence of 1:200 000 worldwide, although this varies between populations (1:60 000 in Ireland). Other causes are various defects of homocysteine remethylation, including homozygosity for 5,10-methylenetetrahydrofolate reductase (*EC* 1.7.99.5; MTHFR) deficiency and errors of cobalamin metabolism. While heterozygotes for these conditions have elevated plasma homocysteine, this is a relatively rare explanation for hyperhomocysteinaemia in population studies.

Kang *et al.* (1988*a,b*) described a thermolabile mutant of MTHFR that occurs in neurologically-normal patients. This defect is inherited recessively and the enzyme appears to have about 50 % of the activity of normal MTHFR and can result in moderate elevation of homocysteine levels in blood (Kang *et al.* 1991*a,b*, 1992). Kang *et al.* (1991*b*) found the thermolabile variant to be present in about 5 % of the general population and 17 % of patients with coronary artery disease. However, many of those with the thermolabile genotype do not have the hyperhomocysteinaemic phenotype, and in the Kang *et al.* (1987) original study, hyperhomocysteinaemia was associated with low plasma folate concentrations; folate supplementation normalizing these levels. Thus, there is thought to be an association between thermolabile MTHFR status, folate and homocysteine levels. Two recent studies have confirmed this. Jacques *et al.* (1996) screened 365 individuals from the NHCBI Family Heart Study. Among individuals with lower plasma folate (< 15.4 nmol/l), homozygous thermolabiles had fasting homocysteine levels that were 24 % greater than individuals with the normal genotype. A difference between genotypes was not seen among individuals with folate levels ≥ 15.4 nmol/l. Harmon *et al.* (1996) looked at 625 working men aged 30–49 years, measuring genotype, plasma homocysteine, serum folate and vitamin B₁₂. The homozygous thermolabile genotype was observed in 48, 36 and 23 % of the top 5, 10, and 20 % of individuals respectively ranked by homocysteine levels, compared with a frequency of 11.5 % in the study population as a whole, establishing that the mutation is a major determinant of homocysteine levels at the upper end of the range. Thermolabile homozygotes had a 9.7-fold risk of being in the top 5 % of the homocysteine distribution, compared with non-thermolabile homozygotes. Both serum folate and vitamin B₁₂ varied with genotype, being lowest in the thermolabile homozygotes. These studies are important in that they indicate that individuals who are homozygous for thermolabile MTHFR may have a higher folate requirement for regulation of homocysteine.

In conclusion, therefore, there is strong support for homocysteine as an independent risk factor for atherosclerosis. Further work in this area should be directed towards developing a better understanding of the interplay between the nutritional and genetic factors which contribute to hyperhomocysteinaemia. Intervention studies with clinical endpoints are also required to assess the effect of strategies designed to lower plasma homocysteine.

SUSCEPTIBILITY OF LDL TO OXIDATION

Increased oxidation of LDL is one mechanism which has been proposed to contribute to the atherogenic potential of homocysteine (Parthasarathy, 1987), and it is currently believed that oxidation of LDL is a key early stage in the development of atherosclerosis. Some reports have suggested the presence of oxidatively-modified LDL in plasma (Itabe *et al.* 1996), but most oxidation is believed to occur in the arterial wall, where LDL may be in a microenvironment in which the antioxidants which normally prevent lipid peroxidation can become depleted. The major nutritional factors which may protect LDL against oxidation include tocopherols, retinol and the carotenoids, ascorbate and the flavonoids. The fatty acid composition of the diet is also an important factor determining the susceptibility of LDL to oxidation, with monounsaturated fatty acids protecting LDL against oxidation (Parthasarathy *et al.* 1990).

Several mechanisms are likely to contribute to LDL oxidation. The four major cell types within atherosclerotic lesions are endothelial cells, smooth muscle cells, macrophages and lymphocytes, each of which can oxidize LDL. Transition metals are potent promoters of free-radical formation, and there is some evidence suggesting that free Cu may be present within atherosclerotic plaques (Smith *et al.* 1992). Oxidation may proceed more rapidly after the introduction of seeding hydroperoxides into the LDL particle by lipoxygenase enzymes (Folcik *et al.* 1995). Regardless of the mechanisms involved, oxidation of polyunsaturated fatty acids within LDL leads to the formation of short-chain aldehydes such as malondialdehyde and 4-hydroxynonenal, which react with key lysine residues on the apoB molecule (Steinberg *et al.* 1989). The modified apoB is no longer recognized by the apoB/E receptor, but instead binds to the scavenger receptor on macrophages or smooth muscle cells, leading to unregulated uptake of cholesterol by these cells and the formation of foam cells. These eventually burst and a fatty streak, the first phase of an atherosclerotic lesion, results.

As well as converting macrophages into cholesterol-laden foam cells, it is now apparent that oxidized LDL may have other diverse biological effects (Steinberg *et al.* 1989). It is chemotactic for monocytes, and once these have entered the arterial wall and differentiated into macrophages, oxidized LDL may inhibit their migratory ability and thus trap them (Daugherty & Rateri, 1993). Oxidized LDL is cytotoxic to various cells, including endothelial cells, because of the lipid peroxidation products it contains (Witztum & Steinberg, 1991). Oxidized LDL also inhibits endothelium-derived relaxing factor, which mediates vaso-relaxation of the coronary arteries in response to agents such as acetylcholine (Rosenfeld, 1991). There is, therefore, a potential role for oxidized LDL in altering vasomotor responses, perhaps contributing to vaso-spasm in diseased vessels. In addition, oxidized LDL is immunogenic; auto-antibodies against various epitopes of oxidized LDL have been found in human serum, and immunoglobulin specific for epitopes of oxidized LDL can be found in lesions (Libby & Hansson, 1991; Salonen *et al.* 1992). Minimally-oxidized LDL has been shown to stimulate the secretion of monocyte chemotactic protein by human aortic, endothelial and smooth muscle cells in culture, increasing the binding of monocytes to cultured endothelial cells (Cushing *et al.* 1990) and

in addition can stimulate the expression and secretion of granulocyte and macrophage colony-stimulating factors in human aortic endothelial cells (Rajavashisth *et al.* 1990). Oxidized LDL, therefore, may be able to induce arterial wall cells to produce chemotactic factors, adhesion molecules, cytokine and growth factors which have an important role in the development of the plaque (Witztum, 1993).

A substantial body of epidemiological evidence supports the hypothesis that antioxidant dietary factors which can inhibit LDL oxidation protect against the development of atherosclerosis. A high intake of antioxidant vitamins, particularly vitamin E, but also vitamins A, C and β -carotene, is associated with reduced CHD mortality (Riemersma *et al.* 1991). Gey *et al.* (1991) found a strong inverse relationship between CHD mortality and dietary vitamin E. In a study of nurses in the USA, heart disease incidence was over one-third less in those with high intakes of vitamin E or β -carotene, and numerous other studies support these findings (Jha *et al.* 1995). The evidence for the water-soluble vitamin C is less strong but there may be a synergistic effect between vitamins C and E (Gey *et al.* 1991; Kagan *et al.* 1992). More recently it has also been suggested that dietary flavonoids protect against IHD.

Numerous intervention studies designed to test the hypothesis that increased antioxidant intake will protect against atherosclerosis are currently in progress. In general, these studies are using supplements of antioxidant vitamins or other antioxidants, and early results have not been encouraging. Probuocol is a lipid-lowering drug with potent antioxidant properties which can substantially inhibit the development of atherosclerosis in animal models (Carew *et al.* 1987). The Probuocol Quantitative Regression Swedish Trial tested the ability of probuocol to inhibit the progression of femoral atherosclerosis in man. After 3 years of follow-up, the probuocol group fared no better than the placebo group (Walldius *et al.* 1994), although this may be because probuocol reduces the HDL2 subfraction in addition to its antioxidant properties (Johansson *et al.* 1995). With respect to antioxidant vitamins, The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Trial in heavy smokers found no reduction in CHD morbidity or mortality during 5–8 years of follow-up (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994) and the Beta-carotene and Retinol Efficacy Trial was ended early when researchers recognized an elevated risk of death from lung cancer in those receiving β -carotene (Omenn *et al.* 1996), although again no beneficial effect on cardiovascular disease was found. The Physicians Health Study followed more than 22 000 US male doctors treated with 50 mg β -carotene or placebo every other day for an average of 12 years (Hennekens *et al.* 1996). The trial was conducted in an exemplary manner and its results would appear to rule out any beneficial effect with such supplementation on cardiovascular disease. The Cambridge Heart Antioxidant Study found that short-term (up to 2 years) supplementation with α -tocopherol (268–537 mg/d) reduced CHD morbidity in patients with a history of cardiovascular disease, but found no benefit in terms of mortality (Stephens *et al.* 1996).

How should we interpret the discordance between epidemiological data and the results so far available from clinical trials? It may be that the duration of clinical trials is much too short to show a benefit, and that antioxidant intake over many years is required to prevent atherosclerosis. In addition, the complex mixture of antioxidant micronutrients found in a diet high in fruit and vegetables may be more effective than large doses of a small number of antioxidant vitamins. This idea is supported by the dramatic benefit of a 'Mediterranean' type of diet in myocardial infarction survivors (Delorgeril *et al.* 1994). Certainly, the possibility that pharmacological supplementation with antioxidant vitamins may be harmful at high doses cannot be discounted, and recommendations relating to the use of such supplements must await the results of studies in progress.

CONCLUSION

It is clear that both hyperhomocysteinaemia and LDL oxidation are associated with CHD and that each of these is in some way linked with vitamin deficiency. Research is by no means complete and at present there is uncertainty as to the effectiveness of vitamin supplementation v. increased dietary intake to reduce these cardiovascular risk factors. Further well-designed trials should lead to effective public health recommendations.

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