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The effects of oral vitamin supplementation on cardiovascular risk factors

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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 14916 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 μmol/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 B_{12}) as a cofactor; the methyl donor is 5-methyltetrahydrofolate. During pathway 2, the vitamin B_6-dependent enzyme cystathionine β-synthase (*EC* 4.2.1.22) catalyses the irreversible condensation of homocysteine with serine to form

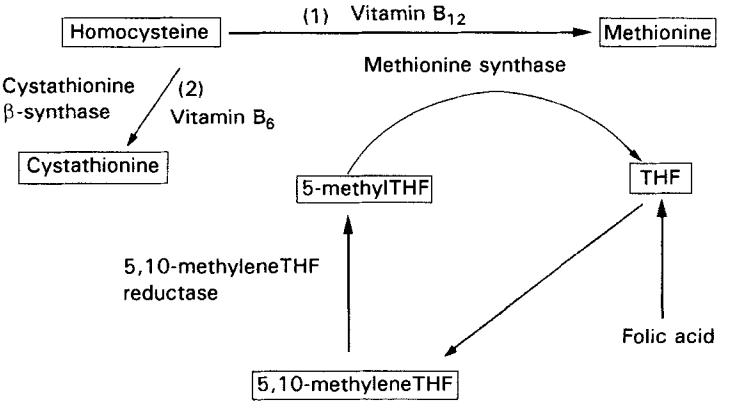

Fig. 1. Metabolism of homocysteine. THF, tetrahydrofolate; methionine synthase, *EC* 2.1.1.13; cystathionine β-synthase, *EC* 4.2.1.22; 5,10-methylene THF reductase, *EC* 1.7.99.5.

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 homocystine, 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 μmol/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 ($< 15{\cdot}4$ 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 $\geq 15{\cdot}4$ nmol/l. Harmon *et al.* (1996) looked at 625 working men aged 30–49 years, measuring genotype, plasma homocysteine, serum folate and vitamin B_{12}. 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 B_{12} 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 immunoglobin 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. Probucol 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 Probucol Quantitative Regression Swedish Trial tested the ability of probucol to inhibit the progression of femoral atherosclerosis in man. After 3 years of follow-up, the probucol group fared no better than the placebo group (Walldius *et al.* 1994), although this may be because probucol 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.

REFERENCES

Alfthan, G., Pekkanen, J., Jauhiainen, M., Pitkaniemi, J., Karvonen, M., Tuomilehto, J., Salonen, J. T. & Ehnholm, E. (1994). Relation of serum homocysteine and lipoprotein(a) concentrations to atherosclerotic disease in a prospective Finnish population based study. *Atherosclerosis* **106**, 9–19.

Arnesen, E., Refsum, H., Bonaa, K. H., Ueland, P. M., Forde, O. H. & Nordrehaug, J. E. (1995). Serum total homocysteine and coronary heart disease. *International Journal of Epidemiology* **24**, 704–709.

Blom, H. J., Kleinveld, H. A., Boers, G. H. J., Demacker, P. N. M., Haklemmers, H. L. M., Poelepothoff, M. T. W. B. T. & Trijbels, J. M. F. (1995). Lipid peroxidation and susceptibility of low-density-lipoprotein to in-vitro oxidation in hyperhomocysteinemia. *European Journal of Clinical Investigation* **25**, 149–154.

Boushey, C. J., Beresford, S. A. A., Omenn, G. S. & Motulsky, A. G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *Journal of the American Medical Association* **274**, 1049–1057.

Brattstrom, L., Israelsson, B., Lindgarde, F. & Hultberg, B. (1988). Higher total plasma homocysteine in vitamin B_{12} deficiency than in heterozygosity for homocystinuria due to cystathionine β-synthase deficiency. *Metabolism* **37**, 175–178.

Carew, T. E., Schwenke, D. C. & Steinberg, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect – evidence that antioxidants in vivo can selectively inhibit low-density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proceedings of the National Academy of Sciences USA* **84**, 7725–7729.

Clarke, R., Daly, L., Robinson, K., Naughten, E., Cahalane, S., Fowler, B. & Graham, I. (1991). Hyperhomocysteinemia: An independent risk factor for vascular disease. *New England Journal of Medicine* **324**, 1149–1155.

Cushing, S. D., Berliner, J. A., Valente, A. J., Territo, M. C., Navab, M., Parhami, F., Gerrity, R., Schwartz, C. J. & Fogelman, A. M. (1990). Minimally modified low density lipoprotein induces monocyte chemotactic protein-1 in human endothelial and smooth muscle cells. *Proceedings of the National Academy of Sciences USA* **87**, 5134–5138.

Cuskelly, G. J., McNulty, H. & Scott, J. M. (1996). Effect of increasing dietary folate on red-cell folate: Implications for prevention of neural tube defects. *Lancet* **347**, 657–659.

Daugherty, A. & Rateri, D. L. (1993). Pathogenesis of atherosclerotic lesions. *Cardiology Reviews* **1**, 157–166.

Delorgeril, M., Renaud, S., Mamelle, N., Salen, P., Martin, J. L. & Monjaud, I. (1994). Mediterranean alpha-linoleic acid-rich diet in secondary prevention of coronary heart disease. *Lancet* **343**, 1454–1459.

Editorial (1984). Progress in coronary artery disease risk-factor research: what remains to te done? *Clinical Chemistry* **30**, 1125–1127.

Finkelstein, J. D. (1990). Methionine metabolism in mammals. *Journal of Nutritional Biochemistry* **1**, 228–237.

Folcik, V. A., Nivar-Aristy, R. A., Krajewski, L. P. & Cathcart, M. K. (1995). Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. *Journal of Clinical Investigation* **96**, 504–510.

Genest, J. J. Jr, McNamara, J. R., Salem, D. N., Wilson, P. W. F., Schaefer, E. F. & Malinow, M. R. (1990). Plasma homocyst(e)ine levels in men with premature coronary artery disease. *Journal of the American College of Cardiology* **16**, 1114–1119.

Gey, K. G., Puska, P., Jordan, P. & Moser, U. K. (1991). Inverse correlation between vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *American Journal of Clinical Nutrition* **53**, Suppl., 326–334.

Giannini, M. J., Coleman, M. & Innerfield, I. (1975). Antithrombin activity in homocystinuria. *Lancet* **i**, 1094.

Harker, L. A., Slichter, S. J., Scott, C. R. & Ross, R. (1974). Homocysteinemia: Vascular injury and arterial thrombosis. *New England Journal of Medicine* **291**, 537–543.

Harmon, D. L., Woodside, J. V., Yarnell, J. W. G., McMaster, D., Young, I. S., McCrum, E. E., Gey, K. F., Whitehead, A. S. & Evans, A. (1996). The common 'thermolabile' variant of methylene-tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. *Quarterly Journal of Medicine* **89**, 571–577.

Harpel, P. C., Chang, V. T. & Borth, W. (1992). Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: A potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proceedings of the National Academy of Sciences USA* **89**, 10193–10197.

Heinecke, J. W., Rosen, H., Suzuki, L. A. & Chait, A. (1987). The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *Journal of Biological Chemistry* **262**, 10098–10103.

Hennekens, C. H., Buring, J. E., Manson, J. E., Stampfer, M., Rosner, B., Cook, N. R., Belanger, C., LaMotte, F., Gaziano, J. M., Ridker, P. M., Willett, W. & Peto, R. (1996). Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *New England Journal of Medicine* **334**, 1145–1149.

Itabe, H., Yamamoto, H., Imanaka, T., Shimamura, K., Uchiyama, H., Kimura, J., Sanaka, T., Hata, Y. & Takano, T. (1996). Sensitive detection of oxidatively modified low-density lipoprotein using a monoclonal antibody. *Journal of Lipid Research* **37**, 45–53.

Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J. & Rozen, R. (1996). Relation between folate status, a common mutation in methylentetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* **93**, 7–9.

Jha, P., Flather, M., Lonn, E., Farkouh, M. & Yusuf, S. (1995). The antioxidant vitamins and cardiovascular disease. *Annals of Internal Medicine* **123**, 860–872.

Johansson, J., Olsson, A. G., Bergstrand, L., Elinder, L. S., Nilsson, S., Erikson, U., Molgaard, J., Holme, I. & Walldius, G. (1995). Lowering of HDL(2B) by probucol partly explains the failure of the drug to affect femoral atherosclerosis in subjects with hypercholesterolemia - a probucol quantitative regression Swedish trial (PQRST) report. *Arteriosclerosis, Thrombosis and Vascular Biology* **15**, 1049–1056.

Kagan, V. E., Serbinova, E. A., Forte, T., Scita, G. & Packer, L. (1992). Recycling of vitamin E in human low density lipoproteins. *Journal of Lipid Research* **33**, 385–397.

Kang, S. S., Wong, P. W. K., Bock, H. G. O., Horwitz, A. & Grix, A. (1991*a*). Intermediate hyperhomocysteinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. *American Journal of Human Genetics* **48**, 546–551.

Kang, S. S., Wong, P. W. K. & Malinow, M. R. (1992). Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Annual Review of Nutrition* **12**, 279–298.

Kang, S. S., Wong, P. W. K. & Norusis, M. (1987). Homocysteinemia due to folate deficiency. *Metabolism* **36**, 458–462.

Kang, S. S., Wong, P. W. K., Susmano, A., Sora, J., Norusis, M. & Ruggie, N. (1991*b*). Thermolabile methylene-tetrahydrofolate reductase: An inherited risk factor for coronary artery disease. *American Journal of Human Genetics* **48**, 536–545.

Kang, S. S., Wong, P. W. K., Zhou, J., Sora, J., Lessick, M., Ruggie, N. & Grcevich, G. (1988*a*). Thermolabile methylenetetra-hydrofolate reductase in patients with coronary artery disease. *Metabolism* **37**, 611–613.

Kang, S. S., Zhou, J., Wong, P. W. K., Kowalisyn, J. & Strokosch, G. (1988*b*). Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. *American Journal of Human Genetics* **43**, 414–421.

Kottke-Marchant, K., Green, R., Jacobsen, D. & Dicorleto, P. (1990). Sub-cytotoxic homocysteine increases monocyte adhesion to human aortic endothelial cells. *Blood* **76**, 511a.

Libby, P. & Hansson, G. K. (1991). Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Laboratory Investigation* **64**, 5–15.

Lindenbaum, J., H.,ealton, E. B., Savage, D. G., Brust, J. C. M., Garrett, T. J., Podell, E. R., Marcell, P. D., Stabler, S. P. & Allen, R. H. (1988). Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *New England Journal of Medicine* **318**, 1720–1728.

McCully, K. S. (1983). Homocysteine theory of arteriosclerosis - development and current status. *Atherosclerosis Reviews* **11**, 157–246.

Malinow, M. R. (1990). Hyperhomocyst(e)ine: a common and easily reversible risk factor for occlusive atherosclerosis. *Circulation* **81**, 2004–2006.

Malinow, M. R., Nieto, F. J., Szklo, M., Chambless, L. E. & Bond, G. (1993). Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults: the Atherosclerosis Risk in Communities Study. *Circulation* **87**, 1107–1113.

Miller, J. W., Ribaya-Mercado, J. D., Russell, R. M., Shepard, D. C., Morrow, F. D., Cochary, E. F., Sadowski, J. A., Gershoff, S. N. & Selhub, J. (1992). Effect of vitamin B_6 deficiency on fasting plasma homocysteine concentrations. *American Journal of Clinical Nutrition* **55**, 1154–1160.

Mudd, S. H., Levy, H. L. & Skovby, F. (1989). Disorders of transulfuration. In *The Metabolic Basis of Inherited Disease*, pp. 693–734 [C. S. Scriver, A. L. Beaudet, W. L. Sly and D. Valle, editors]. New York: McGraw-Hill International Book Co.

Munnich, A., Saudubray, J.-M., Dautzenberg, M.-D., Parvy, P., Ogier, H., Girot, R., Manigne, P. & Frezal, J. (1983). Diet-responsive proconvertin (Factor VII) deficiency in homocystinuria. *Journal of Pediatrics* **102**, 730–734.

Omenn, G. S., Goodman, G. E., Thornquist, M. D., Balmes, J., Cullen, M. R., Glass, A., Keogh, J. P., Meyskens, F. L., Valanis, B., Williams, J. H., Barnhart, S. & Hammar, S. (1996). Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New England Journal of Medicine* **334**, 1150–1155.

Park, K. Y. & Linkswiler, H. (1970). Effect of vitamin B_6 depletion in adult man on the excretion of cystathionine and other methionine metabolites. *Journal of Nutrition* **100**, 110–116.

Parthasarathy, S. (1987). Oxidation of low density lipoprotein by thiol compounds leads to its recognition by the acetyl LDL receptor. *Biochimica Biophysica Acta* **917**, 337–340.

Parthasarathy, S., Khoo, J. C., Miller, E., Barnett, J., Witztum, J. L. & Steinberg, D. (1990). Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. *Proceedings of the National Academy of Sciences USA* **87**, 3894–3898.

Perry, I. J., Refsum, H., Morris, R. W., Ebrahim, S. B., Ueland, P. M. & Shaper, A. G. (1995). Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. *Lancet* **346**, 1395–1398.

Rajavashisth, T. B., Andalibi, A., Territo, M. C., Berliner, J. A., Navab, M., Fogelman, A. M. & Lusis, A. J. (1990). Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature* **344**, 254–257.

Rees, M. M. & Rodgers, G. M. (1993). Homocysteinemia: association of a metabolic disorder with vascular disease and thrombosis. *Thrombosis Research* **71**, 337–359.

Riemersma, R. A., Wood, D. A., Macintyre, C. C. A., Elton, R. A., Gey, K. F. & Oliver M. F. (1991). Risk of angina pectoris and plasma concentrations of vitamins A, C and E and carotene. *Lancet* **337**, 1–5.

Rodgers, G. M. & Conn, M. T. (1990). Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. *Blood* **75**, 895–901.

Rodgers, G. M., Fryer, R. H., Wilson, B. D., Gubler, D. B. & Fitzgerald, L. A. (1993). Homocysteine, a risk factor for vascular disease and thrombosis, induces tissue factor activity in endothelial cells. *Clinical Research* **41**, 19A.

Rodgers, G. M. & Kane, W. H. (1986). Activation of endogenous Factor V by a homocysteine-induced vascular endothelial cell activator. *Journal of Clinical Investigation* **77**, 1909–1916.

Rosenfeld, M. E. (1991). Oxidized LDL affects multiple atherogenic cellular responses. *Circulation* **83**, 2137–2140.

Salonen, J. T., Yla-Herttuala, S., Yamamoto, R., Butler, R., Korpela, H., Salonen, R., Nyyssonen, K., Palinski, W. & Witztum, J. L. (1992). Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet* **339**, 883–887.

Selhub, J., Jacques, P. F., Bostom, A. G., D'Agostino, R. B., Wilson, P. W. F., Belanger, A. J., O'Leary, D. H., Wolf, P. A., Schaefer, E. J. & Rosenberg, I. H. (1995). Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *New England Journal of Medicine* **332**, 286–291.

Selhub, J., Jacques, P. F., Wilson, P. W. F., Rush, D. & Rosenberg, I. H. (1993). Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *Journal of the American Medical Association* **270**, 2693–2698.

Slavik, M., Smith, K. J. & Blanc, O. (1982). Decrease of serum pyridoxal phosphate levels and homocystinemia after administration of pyridozine. *Biochemical Pharmacology* **31**, 4089–4092.

Smith, C., Mitchinson, M., Aruoma, O. I. & Halliwell, B. (1992). Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochemical Journal* **286**, 901–905.

Smolin, L. A. & Benevenga, N. J. (1982). Accumulation of homocyst(e)ine in vitamin B_6 deficiency: A model for the study of cystathionine β-synthase deficiency. *Journal of Nutrition* **112**, 1264–1272.

Smolin, L. A., Crenshaw, T. D., Kurtycz, D. & Benevenga, N. J. (1983). Homocysteine accumulation in pigs fed diets deficient in vitamin B-6: relationship to atherosclerosis. *Journal of Nutrition* **113**, 2022–2033.

Stabler, S. P., Marcell, P. D., Podell, E. R., Allen, R. H., Savage, D. G. & Lindenbaum, J. (1988). Elevation of total homocysteine in serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. *Journal of Clinical Investigation* **81**, 466–474.

Stampfer, M. J., Malinow, M. R., Willett, W. C., Newcomer, L. M., Upson, B., Ullmann, D., Tishler, P. V. & Hennekens, C. H. (1992). A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *Journal of the American Medical Association* **268**, 877–881.

Starkebaum, G. & Harlan, J. M. (1986). Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *Journal of Clinical Investigation* **77**, 1370–1376.

Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witztum, J. L. (1989). Beyond cholesterol: Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine* **320**, 915–924.

Stephens, N. G., Parsons, A., Schofield, P. M., Kelly, F., Cheeseman, K., Mitchinson, M. J. & Brown, M. J. (1996). Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* **347**, 781–786.

Taylor, L. M. Jr, DeFrang, R. D., Harris, E. J. Jr & Porter, J. M. (1991). The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. *Journal of Vascular Surgery* **13**, 128–136.

The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group (1994). The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine* **330**, 1029–1035.

Ubbink, J. B., Vermaak, W. J. H., van der Merwe, A. & Becker, P. J. (1993). Vitamin B12, vitamin B6, and folate nutritional status in men with hyperhomocysteinemia. *American Journal of Clinical Nutrition* **57**, 47–53.

Ubbink, J. B., Vermaak, W. J. H., Van der Merwe, A., Becker, P. J., Delport, R. & Potgieter, H. C. (1994). Vitamin requirements for the treatment of hyperhomocysteinemia in humans. *Journal of Nutrition* **124**, 1927–1933.

Ueland, P. M., Refsum, H. & Brattstrom, L. (1992). Plasma homocysteine and cardiovascular disease. In *Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function*, pp. 183–236 [R.B. Francis Jr, editor]. New York: Marcel Dekker Inc.

Ueland, P. M., Refsum, H., Stabler, S. P., Malinow, M. R., Andersson, A. & Allen, R. H. (1993). Total homocyst(e)ine in plasma or serum: methods and clinical applications. *Clinical Chemistry* **39**, 1764–1779.

Uhlemann, E. R., Tenpas, J. H., Lucky, A. W., Schulman, J. D., Mudd, S. H. & Shulman, N. R. (1976). Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. *New England Journal of Medicine* **295**, 1283–1286.

Verhoef, P., Hennekens, C. H., Malinow, M. R., Kok, F. J., Wilett, W. C. & Stampfer, M. J. (1994). A prospective study of plasma homocyst(e)ine and risk of ischemic stroke. *Stroke* **25**, 1924–1930.

Walldius, G., Erikson, U., Olsson, A., Bergstrand, L., Hadell, K., Johansson, J., Kaijser, L., Lassvik, C., Molgaard, J., Nilsson, S., Schaferelinder, L., Stenport, G. & Holme, I. (1994). The effect of probucol on femoral atherosclerosis: The Probucol Quantitative Regression Swedish Trial (PQRST). *Journal of Cardiology* **74**, 875–883.

Witztum, J. L. (1993). Role of oxidized low-density lipoprotein in atherogenesis. *British Heart Journal* **69**, S12–S18.

Witzum, J. L. & Steinberg, D. (1991). Role of oxidized LDL in atherogenesis. *Circulation* **83**, 1785–1792.