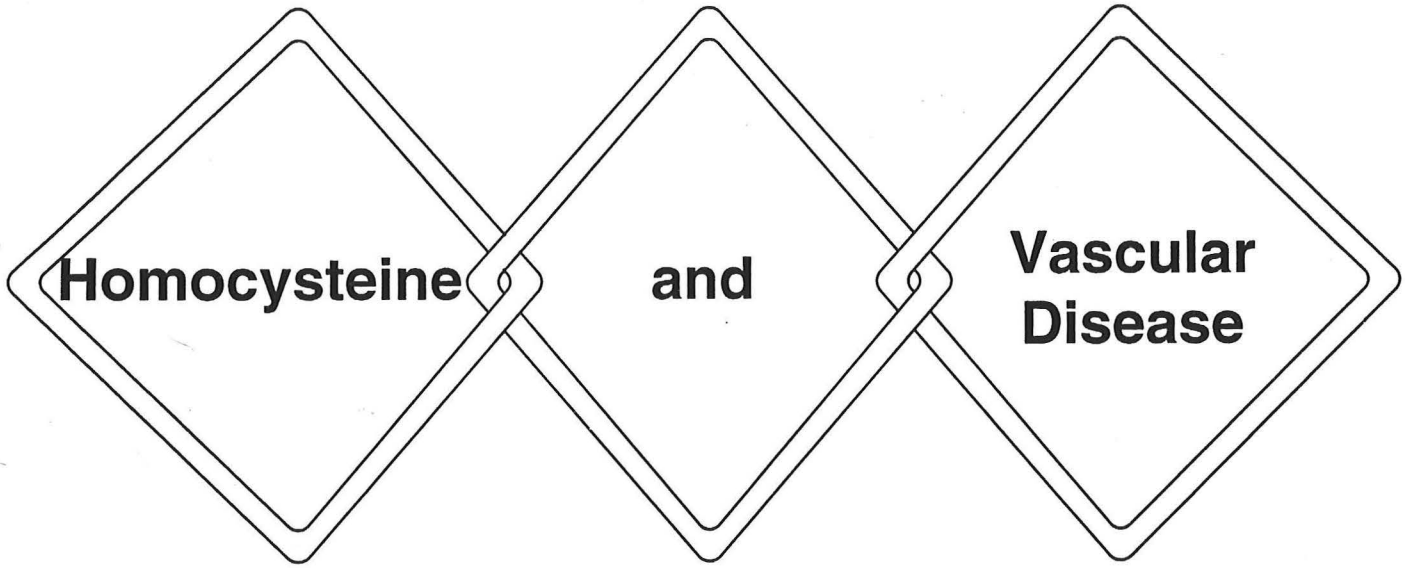
The Link

Between



Helen H. Hobbs

Medical Grand Rounds

4/18/96

INTRODUCTION

Over 25 years ago Dr. Kilmer McCully (Fig. 1), a pathologist at the Massachusetts General Hospital, proposed that elevated plasma levels of homocysteine were associated with the development of vascular lesions (McCully, 1969). He based this conclusion on the autopsy findings of a 7 ½ week old infant who had a very high plasma level of homocysteine due to a rare defect in the vitamin B_{12} metabolic pathway (*cblC*). At autopsy the child's arteries were riddled with what superficially appeared to be rapidly progressive atherosclerotic lesions. Microscopic analysis of the the small, medium and large arteries revealed multiple focal fibrous intimal plaques which extended into, and frequently disrupted, the internal elastic membrane. A branch of the coronary artery is shown in Fig. 2. The lesions differed from the usual atherosclerotic lesion in that they contained very little lipid and were surrounded by an intense proliferation of perivascular connective tissues. The only time McCully had seen similar vascular lesions was in individuals with homocystinuria due to defects in the cystathionine *B*-synthase gene. Based on the strikingly similar pathological appearance of the vascular lesions in these two different inborn errors of metabolism, he reasoned that "since the enzymatic abnormalities in both disorders share certain metabolic consequences, the conclusion has been reached that an elevated concentration of homocysteine, homocystine or a derivative of homocysteine is the common factor leading to arterial damage" (McCully, 1969).

Fig. 1

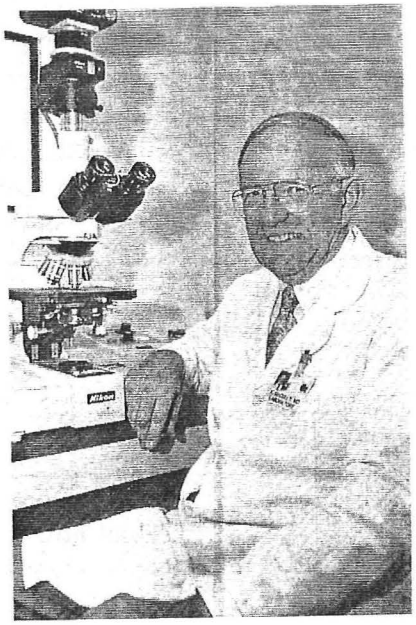
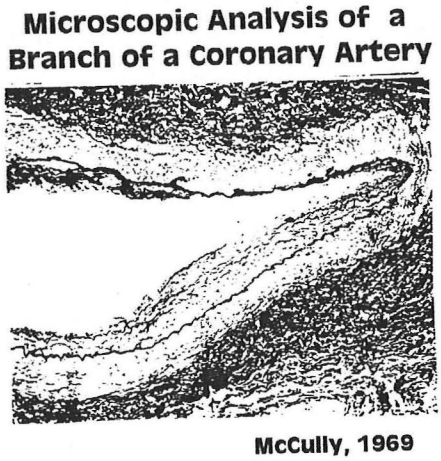


Fig. 2



This theory was supported by the observations that animals fed diets deficient in B_6 or choline, which both are associated with elevated plasma homocysteine levels, had similar arterial lesions. He later went on to propose that more moderate elevations in plasma levels of homocysteine due to subtle defects in the homocysteine metabolic pathway, or to dietary deficiencies in folate or vitamin B_{12}, may be important causes of atherosclerosis in the general population (McCully, 1975).

He pursued his theory for 10 years in the Department of Pathology at Harvard Medical

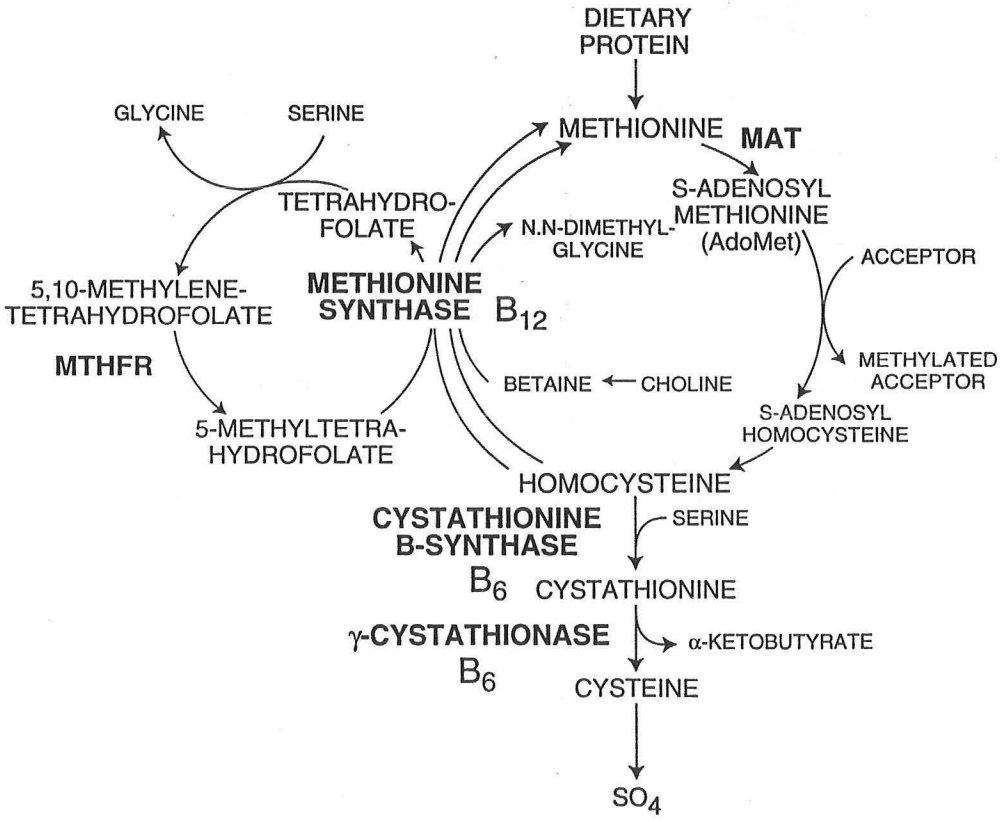
School, but had difficulty convincing his colleagues that homocysteine had anything to do with "garden variety" atherosclerosis. Despite his efforts to develop an animal model with homocysteinemia and vascular disease, he failed to generate enough enthusiasm for his ideas and was dismissed from Harvard Medical School in 1979.

Although the pathophysiological link between high plasma levels of homocysteine and vascular disease remains to be elucidated, it is clear that elevated plasma homocysteine levels are an independent risk factor for coronary artery disease, peripheral vascular disease, cerebrovascular disease and venous thrombosis. Before reviewing the data to support this statement, the biochemical underpinnings of homocystinemia will be briefly reviewed.

BIOCHEMISTRY OF HOMOCYSTEINE SYNTHESIS

The methionine-homocysteine metabolic pathway is shown in Fig. 3 (adapted from Mudd *et al.* 1995). Homocysteine is derived from dietary methionine and is an intermediary metabolite in the cysteine biosynthetic pathway. Cysteine is not an essential amino acid in humans since it can be synthesized from methionine via the transsulfuration pathway. Dietary methionine is converted to s-adenosylmethionine (AdoMet) by the enzyme **methionine-adenosyltransferase** (MAT). MAT forms a high energy sulfonium bond between the 5' ribose carbon of ATP and the sulfur of methionine. A small amount of AdoMet is used to make polyamines, and the remainder donates its methyl group to other compounds (including creatine, DNA and RNA) to form S-adenosylhomocysteine, which in turn is hydrolyzed to homocysteine and adenosine. Individuals homozygous for defects in the gene expressing the hepatic form of MAT have extremely high plasma levels of methionine (250 to 1270 μmol/L; normal < 30 μmol/L) and normal plasma homocysteine levels. These individuals do not have cardiovascular disease (Mudd *et al.* 1995); thus, very high plasma levels of methionine are not associated with vascular disease.

Fig. 3



Homocysteine synthesized from methionine has two possible fates. Approximately 50% of homocysteine is converted irreversibly to cystathionine by the pyridoxal 5′-phosphate (B_6)-dependent enzyme **cystathionine *B*-synthase (CBS),** in the so-called transsulfuration pathway. In this reaction, homocysteine undergoes a condensation with serine to form the thioether, cystathionine. The reaction is allosterically up-regulated by AdoMet, which serves to promote the disposal of excess homocysteine when plasma levels of methionine are high. The next enzyme in the reaction, **γ-cystathionase**, cleaves cystathionine to generate cysteine and **α**-ketobuturate. This enzyme, like CBS, is vitamin B_6-dependent.

The remainder of the homocysteine formed from methionine is remethylated to regenerate methionine. Two different enzymes catalyze this reaction, but the most important is 5-methyltetrahydrafolate-homocysteine methyltransferase (also called **methionine synthase**). This enzyme is found in all cells and requires vitamin B_{12} as a cofactor. The reaction is coupled to the conversion of 5-methyltetrahydrofolate (the circulating form of reduced folate) to tetrahydrofolate, which then enters cells. To generate the 5-methyltetrahydrofolate for this reaction requires the reduction of 5,10-methylenetretrahydrofolate to 5-methyltetrahydrofolate, which is catalyzed by **5,10-methylenetetrahydrofolate reductase (MTHFR).** MTHFR is allosterically inhibited by AdoMet, so high levels of methionine prevent formation of 5-methyltetrahydrofolate (Selhub and Miller, 1992), and thus indirectly, the synthesis of methionine from homocysteine.

Betaine, which is derived from ingested choline, can also serve as a methyl donor in the conversion of homocysteine to methionine. This reaction is catalyzed by betaine-homocysteine methyltransferase, which is another B_{12}-dependent enzyme that is found in significant amounts only in the liver. The relative role of this pathway in the regeneration of methionine in humans is not known.

The conversion of homocysteine to cystathionine or to methionine serves to keep the intracellular concentration of homocysteine low (1-5 μmol/g). Most of the homocysteine in the cell exists in the reduced state (i.e. homocysteine) rather than being linked by a disulfide bond to form a homodimer (homocystine) (Fig. 4) or to other proteins to form mixed disulfides. Excess intracellular homocysteine is exported out of the cell into the plasma.

Fig. 4

Homocysteine *Homocystine*

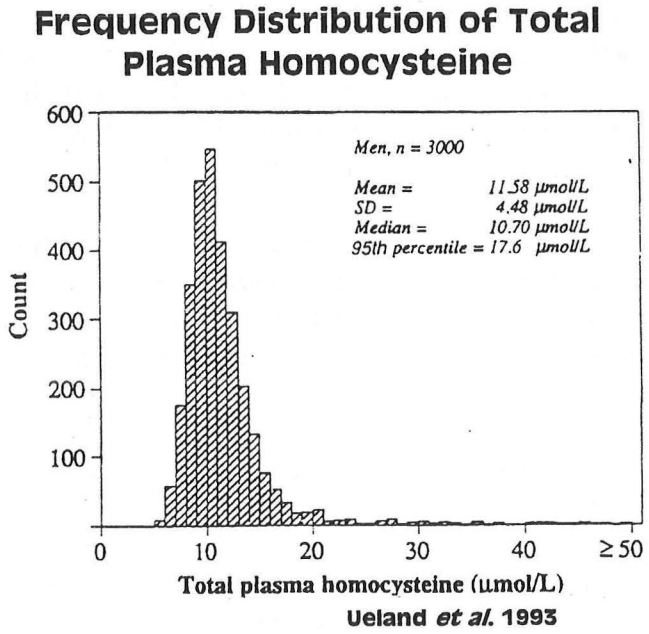
MEASUREMENT OF PLASMA HOMOCYSTEINE

Approximately 70% of the homocysteine that circulates in plasma is protein-bound, mostly to albumin. In normal individuals, the plasma level of total homocysteine ranges from 7 to 14μmol/L

with a mean level of 11.6 μmol/L (Ueland *et al.* 1993) (Fig. 5). Approximately 20-30% of plasma homocysteine is non-protein bound with most of it circulating as homocystine or as homocysteine-cysteine mixed disulfides. Approximately 1-2% circulates as reduced homocysteine. The amount of homocysteine bound to plasma proteins is saturable at about 100 μmol/L of free homocysteine (Ueland *et al.* 1992). Thus, in individuals with very high plasma homocysteine levels (>100 μMol/L), free homocysteine can comprise a significant amount of the total circulating homocysteine (Mudd and Levy, 1995).

Early studies measured only the acid-soluble, non-protein bound fraction of homocysteine, but since the ratio of bound to unbound homocysteine changes with storage, reliable determinations of plasma levels using these methods were problematic. The current methods to measure total plasma homocysteine subject the plasma samples to reduction to release all disulfide linkages prior to quantification of the total amount of homocysteine (Ueland *et al.* 1993). Under these conditions, plasma samples can be stored at -20°C for prolonged periods of time without affecting the level of homocysteine.

Fig. 5



Controversy persists regarding the proper nomenclature to describe "total" homocysteine levels. Mudd and Levy (1995) proposed that when total homocysteine is measured (i.e. protein-bound and unbound), it should be referred to as homocyst(e)ine, since it includes both the free sulfhydryl and disulfide-linked forms. They argue that this is not simply a matter of semantics, since the plasma concentration of the free (reduced) sulfhydryl form of homocysteine increases with increasing amounts of total homocysteine and may be very important in the pathogenesis of vascular lesion formation. This nomenclature, though technically correct, has not been generally accepted and will not be used in this Grand Rounds; plasma homocysteine levels are synonymous with plasma homocyst(e)ine levels in this discussion.

Ideally, the plasma level of homocysteine should be measured in the fasting state. Food consumption results in modest, but significant, lowering of homocysteine levels with a return to

baseline levels in eight hours (Ubbink *et al.* 1992). The blood must be kept on ice from the time it is collected until the plasma can be separated from the cells, which should be performed within four hours. If blood samples are kept at room temperature, continued metabolism of methionine occurs within the red blood cells and in one hour there is a 10% increase in plasma homocysteine level (Ueland *et al.* 1993). The plasma level of homocysteine is stable in plasma at room temperature for days, at 4°C for weeks and at -20°C for years. The level is insensitive to freezing and re-thawing (Ueland *et al.* 1993).

Reliable ranges for normal plasma levels of homocysteine have not been established and good reference standards to calibrate assays in different laboratories are not available. In some studies, men and post-menopausal women have higher plasma homocysteine levels, though this has not been a universal finding (Ueland *et al.* 1993). Children tend to have lower plasma homocysteine levels (Ueland *et al.* 1992) and levels tend to increase with increasing age. Thus, when comparing plasma homocysteine levels between groups, patients must be appropriately age-, sex-, and estrogen-status matched.

HOMOCYSTINURIA

Homocystinuria is a generic term given to the inborn errors of metabolism associated with very high plasma levels of homocysteine and excretion of large amounts of disulfide-linked homocysteine (homocystine) into the urine. Three major genetic disorders result in this phenotype. These disorders will be discussed individually with a focus on their metabolic effects and associated vascular lesions.

Cystathionine *B*-synthase (CBS) deficiency

This autosomal recessive disease is the most common cause of severe homocystinemia and homocystinuria (Mudd *et al.* 1995). The disease was first identified in 1962 when Carson and Neil found two mentally retarded siblings with large amounts of homocysteine in their urine (Carson and Neill, 1962). In 1964, Mudd discovered that the homocystinuria was associated with markedly decreased hepatic CBS activity (Mudd *et al.* 1964). The frequency of CBS homozygotes in North America has been estimated to be 1 in 200,000. Based on neonatal screening programs that measure plasma methionine levels, the world-wide incidence of CBS deficiency is 1 in 344,000, ranging from 1 in 58,000 in Ireland, to 1 in 889,000 in Japan (Mudd *et al.* 1995) (Fig. 5). These screening program underestimate the true incidence of the disease since they fail to detect affected individuals with less severe forms of the disorder.

Fig. 6 **Perinatal Screening for CBS Deficiency by Measuring Plasma Methionine***

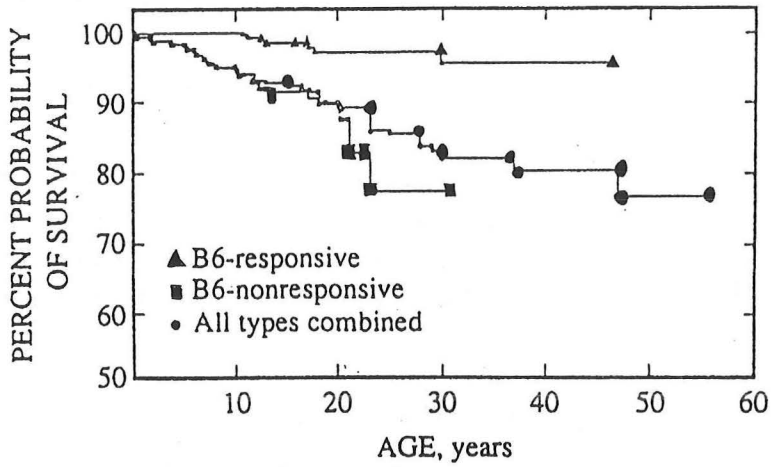
Country	Rate
Ireland	1:58,000
N. Ireland	1:78,000
England	1:126,000
USA	1:291,100
Austria	1:465,000
N. Zealand	1:479,000
Japan	1:889,000
TOTAL	1:344,000

*Often does not detect pyridoxine - responsive form

There is considerable variability in the clinical manifestations of CBS deficiency. In 1967, Barber and Spaeth made the important observation that daily administration of 250 to 500 mg of vitamin B_6 decreases serum methionine levels and eliminates homocystinuria in some affected patients (Barber and Spaeth, 1967). Recall, pyridoxine is a precursor for pyridoxal 5'-phosphate, which is a cofactor for CBS. Approximately 50% of CBS homozygotes improve significantly if given 100 - 1000 mg supplemental vitamin B_6 each day. In addition, folate is required to achieve an optimal response in some vitamin B_6-responsive CBS homozygotes. Individuals with vitamin B_6-responsive CBS deficiency tend to have later onset of symptoms and can sometimes remain asymptomatic until adulthood (Fig. 7). Without exception, if one affected individual in a family is B_6-responsive, so are the other affected members of the sibship, as would be expected if the responsiveness were due to allelic differences in the CBS gene.

Fig. 7

Probability of Survival in CBS Deficiency



The most common clinical features of CBS deficiency (listed in Fig. 8) were discussed in detail in the Medical Grand Rounds by Dr. Rody Cox (1991) so will be only briefly reviewed here. CBS homozygotes have plasma homocysteine levels in excess of 300 μmol/L and can excrete up to 1 mmol of homocysteine a day. They also have very high plasma levels of methionine (up to 2000 μM), low-normal levels of plasma cystathionine, and low levels of plasma cysteine. The most common and characteristic clinical features of the disorder are ectopia lentis, mental retardation, osteoporosis, and arterial and venous thromboembolism.

Fig. 8

COMMON CLINICAL MANIFESTATIONS OF CBS DEFICIENCY

EYE - ectopic lentis
myopia

SKELETAL - osteoporosis
scoliosis, dolichostenomalia
widened metaphyses with spicules
high-arched palate
arachnodactyly

CNS - mental retardation
psychiatric problems
seizures

VASCULAR - vascular occlusions
malar flush
livido reticularis

Ectopia lentis can occur as early as age two, and 90% of affected individuals have dislocated lens by age 20. By age 28, only 6% of affected individuals will not have dislocated lens. The lens is dislocated downward in contrast to subjects with Marfan syndrome, in whom the lens is dislocated superiorly. Disarray of the zonular fibrils that hold the lens in position is the pathological hallmark of eye involvement with this disease. It has been proposed that the high levels of homocysteine (and free sulfhydryl groups) may interfere with the cross linking of fibrillin (the protein that is mutated in Marfan's syndrome) or other cysteine-rich proteins important in the maintenance of the structural integrity of the suspensory fibers.

Mental retardation affects between 40-60% of homozygotes, and affected individuals can also have seizures (20%),extrapyramidal signs, or psychiatric disorders.

Numerous skeletal abnormalities, some resembling those of Marfan syndrome, are found in subjects with CBS deficiency. Affected individuals often have disproportionate growth of their long bones (dolichostenomelia), arachnodactyly, scoliosis, pes canus, and pectus excavatum. A distinctive feature of this disease is the presence of severe, premature osteoporosis, which is due to defective osteoid development and hypomineralization of the bone. A radiographically characteristic lesion, "codfish vertebrae", is a classic feature of the disease.

The most clinically devastating sequelae of this disorder are thromboembolic events. Approximately 50% of the patients experience arterial and/or venous thromboembolism, most commonly involving the cerebral and peripheral circulation (Fig. 9, Mudd *et al.* 1995). An international survey of 629 patients with CBS deficiency revealed that 158 of the sample had experienced 253 events. A quarter of the pyridoxine-nonresponders had their first event before age 16, and 50% of all subjects had an event before age 29 (Mudd *et al.* 1985). The frequency distribution of the different vascular sites of the thromboembolic events were as follows: 51%-peripheral veins, 32%-cerebrovascular, 11%-peripheral arteries, and 4%-coronary arteries. In addition, female patients have a higher than normal rate of fetal loss, presumably due to thrombosis at the implantation site.

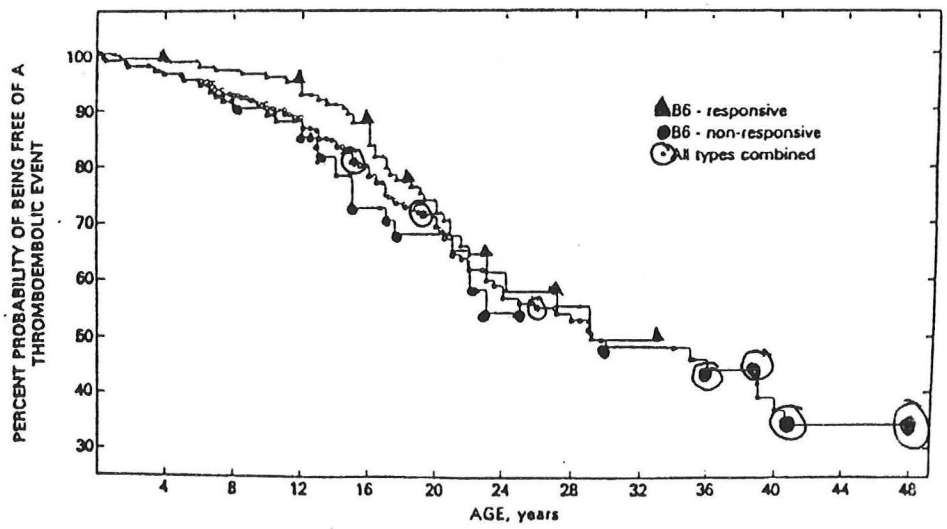


Fig. 9. Probability of being free of thromboembolic event in untreated CBS-deficient subjects.

On pathological evaluation at autopsy, multiple thrombi and emboli are frequently present throughout the vasculature, including the dural sinuses. There is generalized fibrous thickening in the walls of the large and medium sized arteries. The intima can become so thick that it blocks the lumen of some vessels. The elastic lamina is frequently frayed with disarray of the elastic fibers. Smooth muscle fibers in the media are disorganized, and there is a generalized increase in collagen and cystic material in the vessel wall. A distinctive feature of the arterial lesions is the paucity of lipid deposition.

The treatment of CBS deficiency includes high-dose pyridoxine in responsive-patients, a methionine-restricted diet (with additional cysteine), and supplementation with betaine and folate to promote the methylation of homocysteine.

Molecular genetics of CBS deficiency

Jan Kraus and his colleagues cloned and characterized the human CBS cDNA and gene, which is located on the long arm of chromosome 21 at band 22.3 in a region thought to contain the genes responsible for many of the phenotypic effects of Trisomy 21 (Kraus *et al.* 1993; Kraus, 1994). The gene is 20-25 kilobases in length and has 17 exons. The coding regions of the rat and human CBS genes are 90% identical in sequence, and share 52% sequence identity with O-acetylserine(thiol)-lyase (or cysteine synthase) of bacteria and plants (Kraus *et al.* 1993). The CBS gene encodes a 551 amino acids, 63 kDa protein that forms an inactive tetramer in cells. The inactive tetrameric form of the enzyme is converted to an active dimeric 48 kDa form by limited proteolysis, and only 5% of the immunoreactive CBS protein in the liver is in the dimeric, active form. The mechanism responsible for the conversion of the inactive, to the active, form of CBS and its regulation are not known.

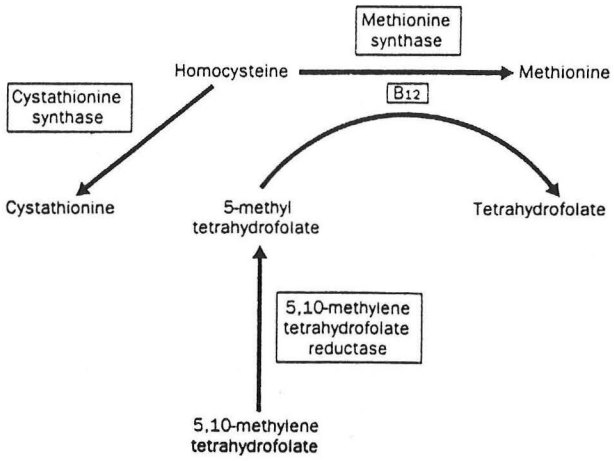
At least 16 mutations have been identified in the CBS gene (Kraus, 1994; Kluijtmans *et al.* 1995). Most of the mutations are missense mutations that cluster in the N-terminal half of the cDNA. A few mutations are frequent in particular populations, presumably due to founder effects. For example, in the Netherlands, 50% of all the mutant alleles have the same mutation (I278T). A common Celtic mutation (G307S) accounts for 71% of the mutant alleles in Ireland, where the disease has the highest frequency in the world (Gallagher *et al.* 1995). This mutation is a frequent cause of CBS deficiency among individuals of Celtic ancestry in the USA and Australia and is not associated with vitamin B_6 responsiveness.

5,10 Methylenetetrahydrofolate reductase (MTHFR deficiency)

A much less frequent inborn error of metabolism associated with homocystinuria is severe MTHFR deficiency. MTHFR catalyzes the NADPH-linked reduction of 5,10 methylenetetrahyhrofolate to 5-methyltetrahydrofolate (Fig. 10) (Rosenblatt, 1995). It is a flavoprotein that transfers electrons to NADP. The enzyme is inhibited by AdoMet, so at high methionine levels the formation of 5-methyltetrahydrofolate, and thus methionine, is limited. The gene has been localized to 1p36.3 (Goyette *et al.* 1994) and the N-terminal region shares homology with the *met F* gene of *E. coli*, which binds and catalyzes the same reaction. To date, nine different

mutations have been identified in the MTHFR gene that cause the severe form of the disease (Goyette *et al.* 1995).

Fig. 10



More than 30 patients with severe deficiency in MTHFR activity (0-20%) have been described (Rosenblatt, 1995). This disorder presents in infancy or adolescence and affected individuals, like subjects with CBS deficiency, have psychiatric and neurological problems, including developmental delay, gait disorders, and seizures. They also have vascular lesions that are pathologically indistinguishable from those seen in individuals with CBS deficiency (Kanwar *et al.* 1976). Treatment with betaine promotes synthesis of methionine from homocysteine through the alternative pathway and benefits some patients.

Plasma levels of homocysteine are not as high in homozygotes with MTHFR deficiency as in subjects with CBS deficiency (mean ~ 50 vs. 300 μmol/L), and the plasma levels of methionine are normal and folate levels low in these individuals. MTHFR-deficient subjects can be distinguished from individuals with defects in vitamin B_{12} metabolism by the absence of methylmalonic acidemia and megaloblastic anemia. The defect can be detected by assaying enzyme activity in fibroblasts and lymphocytes (Rosenblatt, 1995).

Another much more common, and less severe, form of MTHFR deficiency is due to a mutation that results in thermolability of the enzyme (Kang *et al.* 1988). Homozygotes for thermolabile MTHFR have ~50% of normal MTHFR activity in lymphocytes extracts. If the enzyme is heated to 46° C for 5 min, it retains only 7-17% of its activity after cooling (normal enzyme: 20-50%). This phenotype is due to a C to T transition in at codon 677, which substitutes a valine for an alanine (Frosst *et al.* 1995). The corresponding region in the *Met F* gene of *E. coli* binds folate, which may stabilize the enzyme. The frequency of homozygotes is ~ 5% in most populations and their plasma homocysteine levels range from 4.3 to 38 μmol/L (Kang *et al.* 1991). Thus, homozygosity for this mutation is not necessarily associated with high plasma levels of homocysteine. The methionine loading test (which will be reviewed later) is often abnormal in these patients, even if the fasting plasma level of homocysteine is normal (Engbersen *et al.* 1995). The French Canadian population has a very high frequency of the thermolabile allele (0.38%); 12% of blood donors were found to be homozygous for the defect (Frosst *et al.* 1995). In this population, the homozygotes have a mean plasma level of homocysteine that is twice the level of normal controls (22.4 +/-9 vs.

12.6 +/-1.1 μmol/L).

The high frequency of this mutation begs the question as to whether there is a survival advantage associated with heterozygosity for the MTHFR thermolabile allele. It has been proposed that perhaps during starvation this defect diverts the use of the methyl groups of 5-methyltetrahydrofolate away from the synthesis of methionine to the more essential formation of purines and thymidine (Engbersen *et al.* 1995).

Defects in the formation of methyl-cobalamin

Since methionine synthase is a methylocobalamin-dependent enzyme, defects in the generation of methylcobolamin are also associated with very high plasma levels of homocysteine. Several genetic defects in the B_{12} metabolic pathway have been described (Fenton and Rosenberg, 1995) but a detailed description of these rare genetics disorders, and how they can be distinguished clinically from the other cause of homocystinuria, is beyond the scope of this review. Important for this discussion is the fact that these individuals have high plasma homocysteine levels, normal plasma methionine levels and often die from thromboembolic complications (Ueland *et al.* 1992). At autopsy vascular lesions similar to those found in the other forms of homocystinuria are often present.

PATHOPHYSIOLOGICAL LINK BETWEEN HOMOCYSTINEMIA AND VASCULAR LESIONS

Since McCully first implicated high plasma levels of homocysteine as a risk factor for vascular lesion development (McCully, 1969), investigators have struggled to make the pathophysiological link between high plasma homocysteine levels and vascular disease.

Animal models and endothelial injury

Numerous attempts have been made to develop an animal model to study the effects of homocysteine on the vasculature. Some of the very early, less well-characterized models were used by McCully to support his original hypothesis that high plasma homocysteine levels cause vascular lesions (McCully, 1969). The problem with these animal studies is that the plasma levels of homocysteine were not measured.

In 1976 Harker published a provocative paper in the Journal of Clinical Investigation in which he reported that chronic infusion of homocysteine thiolactone into baboons resulted in a ~ 8% loss of aortic endothelial cells (Harken *et al.* 1976). At autopsy, the animals had prominent fibromuscular lesions throughout their vasculature. Associated with the development of vascular lesions was an increase in platelet turnover, which could be inhibited by treating the baboons with dipyridamole. He also reported a similar dipyridamole-inhibitable increase in platelet turnover in individuals with CBS deficiency (Harker *et al.* 1974). There are a number of problems with these frequently cited studies. First, homocysteine thiolactone is not a naturally circulating form of homocysteine and has not been reproducibly identified in the serum of CBS-deficient individuals. Second, the enhanced platelet

turnover and the positive effect of dipyridamole seen by Harker could not be replicated by other investigators (Uhlemann *et al.* 1976; Hill-Zobel, *et al.* 1982). Finally, tissue culture studies supporting a toxic effect of homocysteine on the endothelium either used homocysteine thiolactone, or nonphysiologic levels of homocysteine in the culture media (Wall *et al.* 1980). [Recall, only ~ 1% of the homocysteine that circulates in plasma is in the free sulfhydryl, reduced form so addition of 1 mM homocysteine results in a concentration of homocysteine that is 5,000 times higher than found in the plasma of CBS-deficient individuals (Blom and Van der Moler, 1995).]

Another leading hypothesis is that the oxidation of homocysteine to homocystine, which is promoted by copper, is associated with the generation of hydrogen peroxide and thus, secondary endothelial injury. The concentrations of homocysteine required to demonstrate these effects in tissue culture are much are much higher than those seen in individuals with CBS deficiency (Starkebaum *et al.* 1986). In some hands, homocysteine can oxidize the lipids of low density lipoproteins (LDL) (Heinecke *et al.* 1987), but the significance of this observation is questionable since the plasma levels of lipid peroxidation products in the CBS-deficient subjects have either been normal or even decreased (Blom *et al.* 1992). It would be of interest to determine if anti-oxidixed LDL antibody titers are elevated in homocystinuric subjects.

Platelet dysfunction

The large number platelet-filled thrombi in the vasculature of individuals with CBS deficiency has motivated many investigators to search for homocysteine-induced defects in platelet function. Early reports of defects in platelet aggregation and adhesion in CBS deficient individuals have not been confirmed (Di Minno *et al.* 1993; Stamler *et al.* 1993). The only convincing data suggesting a possible defect in platelet metabolism is the finding of an 5-fold increase in the excretion of urinary metabolites of thromboxane (TX) A_2 , a potent potentiator of platelet aggregation, in CBS-deficient individuals (control : 345 +/- 136 pg/mg creatinine vs CBS-deficient: 1724 +/- 828 pg/mg creatinine) (Di Minno *et al.* 1993). Definition of the mechanism by which homocysteine mediates these changes in TXA_2 synthesis may provide a much needed molecular handle on the cellular effects of homocysteine accumulation.

Effects on hemostasis

Several studies have examined the effect of homocysteine on the function of factors important in hemostasis. In most of these studies, homocysteine was added to the media of cultured endothelial cells, and the synthesis and/or function of the target proteins was assayed. Almost without exception, no effect was seen unless the concentration of homocysteine added to the media greatly exceeded that found in patients with homocystinuria. For example, incubation of endothelial cells with 2.5 **mmol** homocysteine is associated with a 70% decrease in thrombomodulin co-factor activity and a 50% decrease in thrombin binding (Hayashi *et al.* 1992). (Remember, plasma levels of homocysteine in CBS deficiency are only 0.3 mmol). Others have shown that transport of thrombomodulin and endothelial relaxation factor to the cell surface is impaired, but the effect is only

seen if astronomically high levels of homocysteine are added in the media (Lentz and Sadler, 1991). At more reasonable, but still very high, levels of homocysteine (0.2 mmol/L), the processing and secretion of Von Willebrand factor from the endoplasmic reticulum is reduced (Lentz and Sadler, 1993). Others have shown decreases in the number of endothelial binding sites for t-PA (Haijar, 1993) and the synthesis of antithrombin III and tissue factor by cultured endothelial cells (Fryer *et al.* 1993), but in all these studies extremely high levels of homocysteine were needed to see the effects.

Interaction with Lp(a)

Homocysteine may potentiate the atherogenic effects of lipoprotein(a) [Lp(a)] a particle composed of a low density lipoprotein particle and a large glycoprotein called apolipoprotein(a) [apo(a)] (Harpel *et al.* 1992). Lp(a) binds immobilized fibrin with high affinity *in vitro*, especially if the fibrin is pretreated with plasmin. Incubation of the Lp(a) with homocysteine in concentrations as low as 8 μmol/L are associated with a significant increase in binding of Lp(a) to fibrin. It would be of interest to perform immunolocalization studies to determine if there is Lp(a) in the vessel wall of homocystinuric subjects.

Mouse model of CBS deficiency

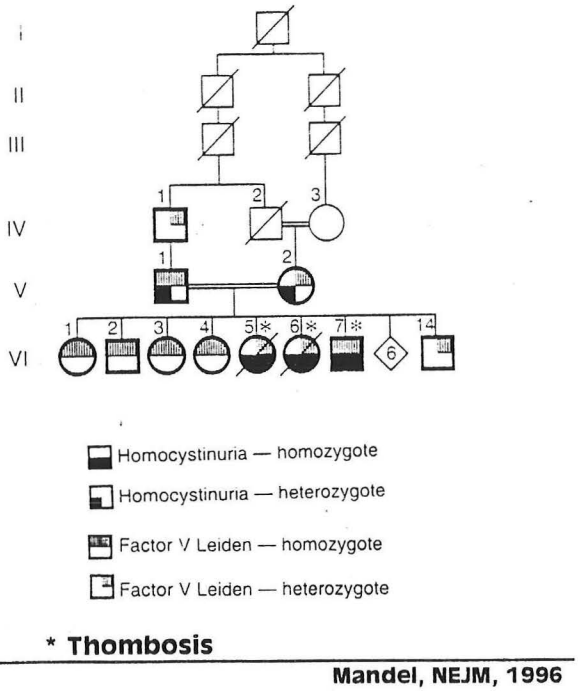
Thus, to date, no convincing data are available regarding the pathogenesis of homocysteine-associated lesion development. Without such data, there can be no certainty that the high levels of plasma homocysteine are directly responsible for lesion development. Recently, a genetically-engineered mouse line was developed in which the CBS gene was inactivated (Watanabe *et al.* 1994). The homozygous mice have very high plasma levels of homocysteine and die in infancy, but the heterozygotes survive into adulthood and have twice the normal plasma homocysteine level. It is not known if these mice develop vascular lesions, but if they do, they will provide an important animal model in which to study the effects of homocystinemia on lesion development. These animals should be bred with other stains of genetically-modified mice that have defects in genes known to be associated with atherosclerotic vascular lesions to look for interactive effects on lesion development.

HOMOCYSTINURIA AND VASCULAR COMPLICATIONS

Only some individuals with homocystinuria have thrombotic complications. Even within families, there is variability in the frequency of thromboembolic events, suggesting that factors other than the gene defect contribute to the development of vascular lesions. Mandel and his colleagues have proposed that co-inheritance of other genes that predispose to thrombosis may be required for the development of clinically-apparent thromboembolism (Mandel *et al.* 1996). To test this hypothesis, the plasma levels and function of protein C, protein S, antithrombin III, and factor V were measured in 7 unrelated Arab-Israeli families with either CBS deficiency (n=3), the severe form of MTHFR deficiency (n=3), or a defect in vitamin B_{12} metabolism (n=1).

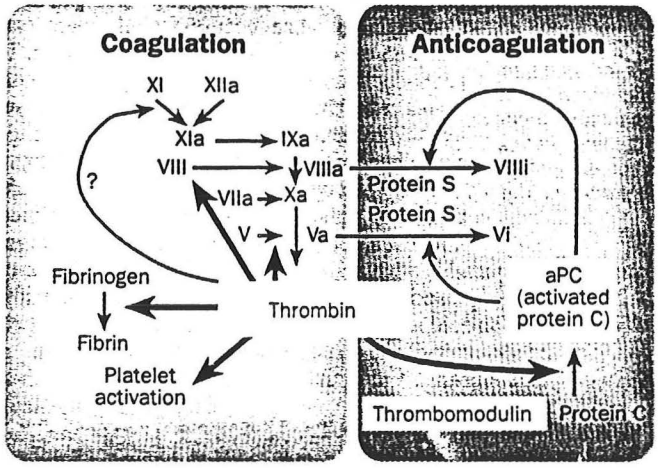
Of the 11 individuals with homocystinuria, thrombotic events were restricted to the 6 individuals who were either heterozygous or homozygous for the factor V-Leiden allele. An example of one of the families is shown in Fig. 11.

Fig. 11. Family with CBS deficiency and thrombo-embolic disease



Factor V-Leiden is the most frequency genetic defect associated with thrombophilia (the tendency to form thrombi) (Dahback, 1995). Normally, protein C is activated on endothelial cells by thrombomodulin-modified thrombin. Activated protein C then inactivates factors Va and VIIIa. Factor V-Leiden has a missense mutation at the site of protein C cleavage (Fig. 11). Between 3-7% of the Caucasian population and 30-60% of individuals with thrombophilia are heterozygous for factor V-Leiden. Heterozygotes have a 5-10 fold, and homozygotes a 50-100 fold, increase in risk for having a thrombotic event.

Fig. 12.



None of the 16 CBS and MTHFR carriers who did not have Factor V-Lieden had a history of thrombotic disease or fetal loss, but three of the MTHFR heterozygotes who were also heterozygous for a factor V-Leiden allele had a high rate of fetal loss (Mandel *et al.* 1995). Though there are reports of an association between high plasma homocysteine levels and recurrent fetal loss (Wouters *et al.* 1993), this was the first study to show that carriers for these severe enzymatic defects may be at increased risk for fetal loss if they also have inherited the factor V-Leiden allele. More studies are needed to confirm this association, and to analyze the relative contribution of each genetic defect to fetal loss.

While these results need to be confirmed in larger patient populations, they strongly suggest that individuals with homocystinuria should be screened for the factor V-Leiden mutations and special precautions should be taken with these individuals, especially before surgical procedures.

ARE HETEROZYGOTES FOR CBS DEFICIENCY AT INCREASED RISK FOR VASCULAR DISEASE?

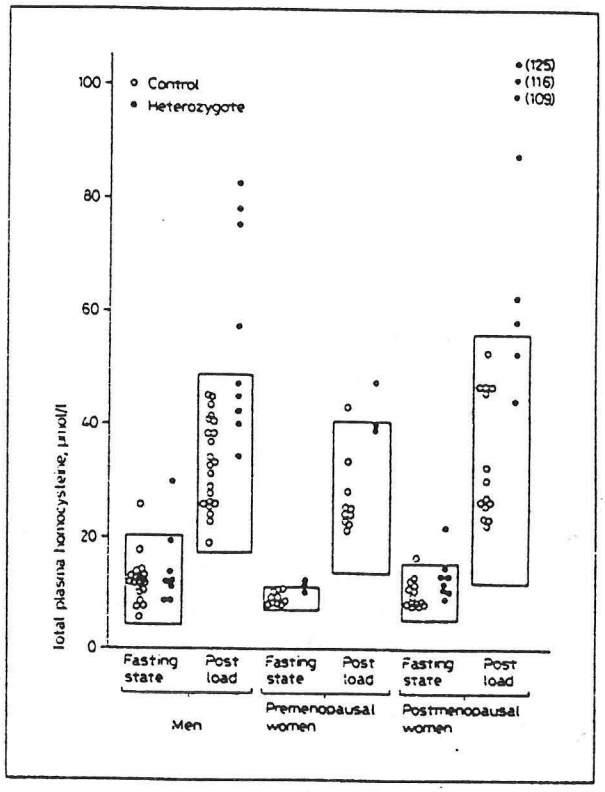
If very high plasma levels of homocysteine are associated with vascular disease, do more modest elevations in plasma homocysteine levels have a pathologic effect, as was proposed originally by McCully? Due to the rarity of severe MTHFR deficiency and defects in cobalamin metabolism, it was thought that CBS deficiency was the only genetic disorder that occurred with sufficient frequency to possibly be an important etiological factor in the development of vascular disease in the general population. If the frequency of CBS deficiency is 1:200,000 worldwide, then the frequency for heterozygosity for this disorder would be ~1 in 220. Are these individuals at a higher risk for the development of vascular disease?

A method to diagnose CBS heterozygotes was required to answer this question. Obligate heterozygotes, i.e. the parents of homozygous offspring, have been appropriately used to define the sensitivity and specificity of each assay developed.

Assays to detect CBS carriers

Though plasma levels of homocysteine tend to be slightly higher than normal in obligate CBS heterozygotes, they can not be used to reliably diagnosis heterozygotes, due to overlap in the range of levels between the groups (Fig.14). CBS is expressed in fibroblasts but assays of CBS activity in cultured cells also fail to differentiate CBS heterozygotes from normals. Obligate heterozygotes tend to have less than 50% of normal CBS activity, but there is significant overlap between the range of values in obligate heterozygotes and normals. A similar overlap is seen when CBS activity is measured in liver extracts and cultured or stimulated lymphocytes (McGill *et al.* 1990; (Goldstein, J.L. *et al.* 1973).

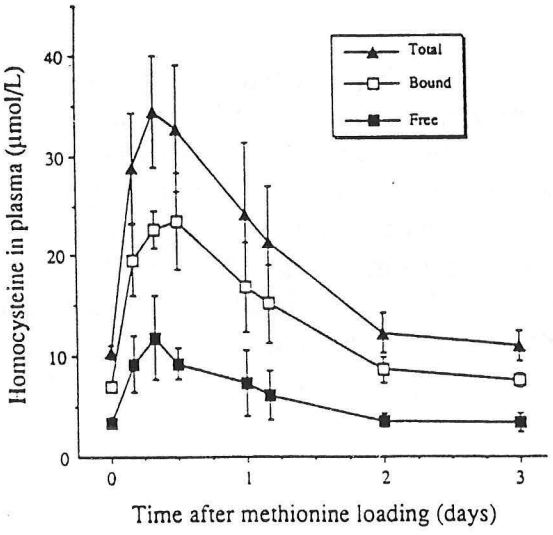
Fig. 13. Plasma homocysteine before and 5-h post methionine load in normal (o) and obligate CBS heterozygotes (•).



Brattström et al. 1989

The methionine loading test was developed in hopes that stressing the methionine-homocysteine metabolic pathway would reveal differences between CBS heterozygotes and normals. In this test, 100 mg/ kg of methionine is administered orally and the plasma levels of homocysteine (and sometimes methionine) level is measured, usually after 4-6 hours (Fig. 14). The test is considered abnormal if the post-methionine plasma homocysteine level is greater than the 95th percentile, or greater than the mean plus two standard deviations, of the controls. Most of the post-methionine plasma homocysteine probably comes from the liver (Ueland *et al.* 1992). CBS heterozygotes tend to have a higher and more prolonged increase in plasma homocysteine levels after the challenge. Early reports suggested that the results of this test were a good predictor of CBS carrier status. In support of this contention, Boer and his colleagues reported that 13 of the 14 of the subjects with an abnormal methionine test had decreased CBS enzyme activity in cultured fibroblasts (Boer *et al.* 1985). Unfortunately, later studies found the methionine-loading test was positive in only 60% of obligate heterozygotes (Ueland *et al.* 1992).

Fig. 14 Plasma homocysteine levels post methionine load.



Ueland *et al.* 1992

Thus, before the development of molecular assays to detect CBS mutations directly, the diagnosis of heterozygous CBS could not be accurately ascertained using either the plasma level of homocysteine or the results of the methionine loading test.

CBS heterozygotes and vascular disease

As imperfect as these tests were for diagnosing CBS heterozygotes, they were used to analyze many subjects with vascular disease. The report that set off a flurry of studies was a paper by Wilchen and Wilchen (1976) who performed the methionine loading test in men less than age 50 who had angiographic evidence of CAD and no cardiac risk factors. A total of 7 of the 25 patients (28%) and only one of the 22 control (5%) had peak plasma homocysteine-cysteine levels that were greater than 10 μmol/L (Fig.15).

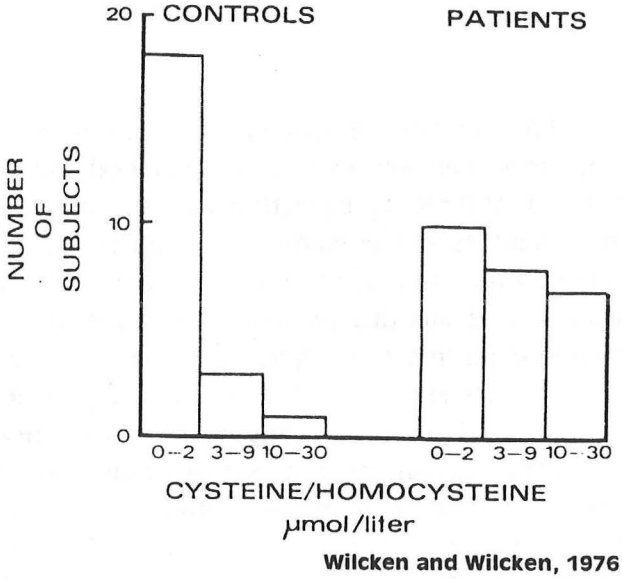
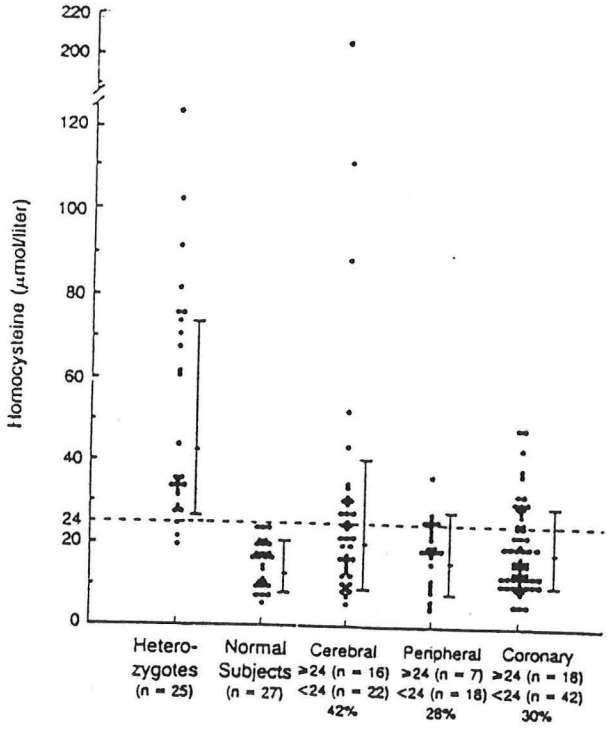


Fig. 15 Post methionine load cysteine-homocysteine levels in control (n=22) and coronary (n=25) patients.

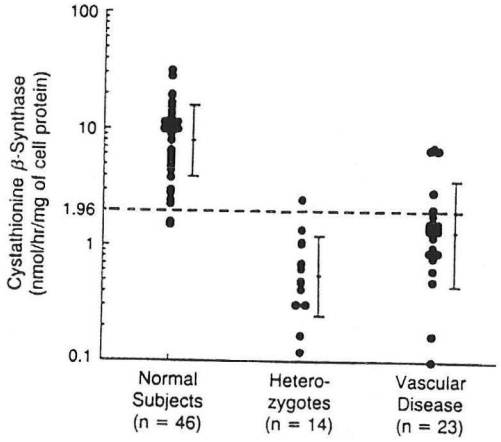
An equally provocative study was performed by Clarke and his colleagues who reported in the New England Journal of Medicine that the methionine loading test was abnormal in 42% of subjects with cerebrovascular disease, 28% with peripheral vascular disease and 30% with cardiovascular disease (Clark, *et al.* 1991) (Fig. 16). The frequency of abnormal tests was 12-fold high in the subjects with vascular disease than controls. Moreover, there was very little overlap in the range of post-methionine plasma homocysteine levels in 25 CBS heterozygotes and normals, and almost 18 of the 23 individuals with an abnormal methionine loading test had reduced CBS activity in cultured fibroblasts (Fig. 17). In this study, the authors claimed that a post-methionine plasma level of homocysteine of 24 μmol/L was 92% sensitive and 100% specific for CBS deficiency.

Fig. 16 Peak post-methionine plasma homocysteine levels in patients with vascular disease.



Clarke, NEJM, 1991

Fig. 17 Cystathionine synthase activity in fibroblasts from individuals with hyperhomocysteinemia and vascular disease.



Clarke, NEJM, 1991

The results of these studies led many authors to concluded that heterozygosity for a CBS mutation was an important cause of vascular disease. However, evidence is now mounting that CBS carrier status is <u>not</u> a risk factor for vascular disease. A survey of 394 parents and 776 grandparents (mean age: men 44, women 41) in 203 families of individuals with CBS deficiency revealed that the incidence of MI or strokes was similar to a control population of achondroplastic dwarfs and individuals with mild phenylalaninemia (Mudd *et al.* 1981). Though the study was relatively small,

and the population of CBS heterozygotes relatively young, the study convincingly demonstrated that heterozygotes for CBS gene defects have no increase in symptomatic vascular disease before age 50.

Two studies have screened for asymptomatic vascular lesions in obligate heterozygotes using sonography to examine the carotid arteries. One study showed a slightly higher incidence of vascular lesions in obligate CBS heterozygotes (Rubba *et al.* 1990), whereas the other found no statistically significant increase in lesions (Clarke *et al.* 1990).

Now that the CBS gene had been cloned, it has been possible to examine the gene sequence directly to determine if gene defects are more common in individuals with vascular disease. Two such studies have been performed. Not a single Dutch individual with premature cardiovascular disease (CAD) (n=60) was heterozygous for a CBS mutation that comprises 50% of the mutant CBS alleles in the Dutch population (Kluijtmans *et al.* 1995). Moreover, the authors were unable to reproduce the findings of Boer *et al.* (1985) and Clarke *et al.* (1991) that subjects with homocystinemia and vascular disease have low CBS activity in fibroblasts using the exact same protocol in the same laboratory (Kluijmans *et al.* 1995). In the other study, 100 Irish individuals with CAD were screened for the common Celtic mutation in the CBS gene (which comprises 70% of the mutant CBS alleles in that population) and no subjects had the mutation (Whitehead *et al.* 1994). The results of this latter study are more significant since the frequency of CBS carriers within this population is ~1 in 50. Finally, in the previously discussed study of 7 Arab-Israeli families with homocystinuria, none of the 16 CBS or MTHFR heterozygotes had a history of either venous or arterial thrombosis (Mandel *et al.* 1996).

More studies need to be performed to determine the frequency of CBS heterozygosity in individuals with various types of vascular disease, but based on recent studies, defects in the CBS gene appear not to be a major contributing factor to CAD.

Though heterozygosity for a CBS mutation may not predispose to vascular disease, a consequence of the search for such a connection was that the plasma homocysteine levels was measured in a large number of individuals with coronary, peripheral and cerebrovascular disease. Repeatedly, individuals with vascular disease tended to have higher plasma levels of homocysteine than did age- and sex-matched controls.

LINK BETWEEN HOMOCYSTINEMIA AND VASCULAR DISEASE

Over 21 case-control and cross-sectional studies involving over 1500 patients with various have examined the relationship between plasma levels of homocysteine and vascular disease; in 16 of these studies such an association was found (Ueland *et al.* 1992). The relationship between hyperhomocystinemia and the various forms of vascular disease will be discussed separately, since the strength of the association differs depending on the site of the vascular lesions.

Cerebrovascular (CVD) disease

In patients with CBS deficiency, the most common symptomatic arterial vascular events involve the cerebrovascular circulation. The first studies to show a relationship between hyperhomocystinemia and CVD used the methionine loading test. In most of these studies, a significantly higher incidence of abnormal tests were obtain in the CVD group, especially in the subset of individuals with early-onset disease (for review, see Ueland *et al.* 1992). The largest study to date measured fasting plasma homocysteine levels in 142 individuals who had suffered a stroke, 57 of these patients (40%) had hyperhomocystinemia, compared to 6% of a poorly matched control group (n=60) (Brattsom *et al.* 1992). Unexpectedly, there was also a significant increase in plasma homocysteine levels in individuals who had hemorrhagic and embolic strokes, as well as those who had suffered a thrombotic stroke.

Several retrospective and prospective studies have been performed to analyze the relationship between CVD and hyperhomocystinemia (for review, see Boushey *et al.* 1995). Meta-analysis of these studies revealed an odds ratio of 2.5 [95% confidence intervals (CI) of 2.0 to 3.0] for CVD being associated with high plasma levels of homocysteine (Fig. 18). If only those studies in which fasting homocysteine levels were measured are included, the odds ratio is 2.3. A five μmol/L increase in homocysteine is associated with an odds ratio of 1.9, and this falls to 1.5 if only very high quality studies are included (Fig. 19). In none of these studies was there any relationship between plasma homocysteine and other risk factors for CVD.

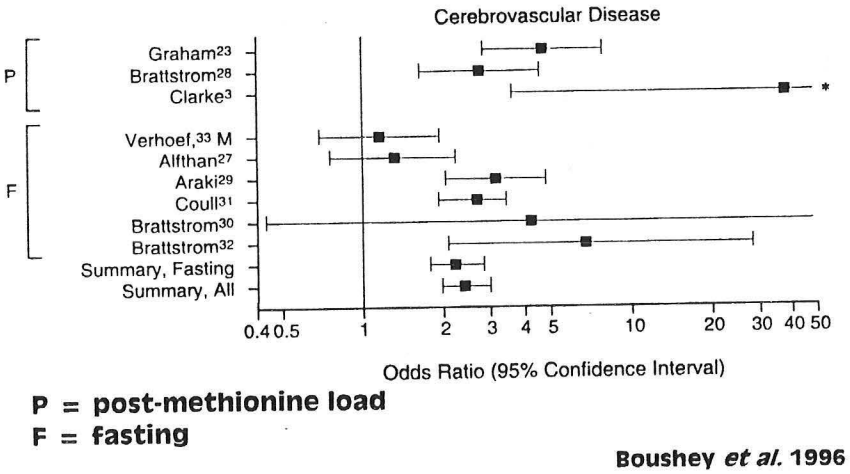


Fig. 18 Odds ratio associated with elevated plasma homocysteine.

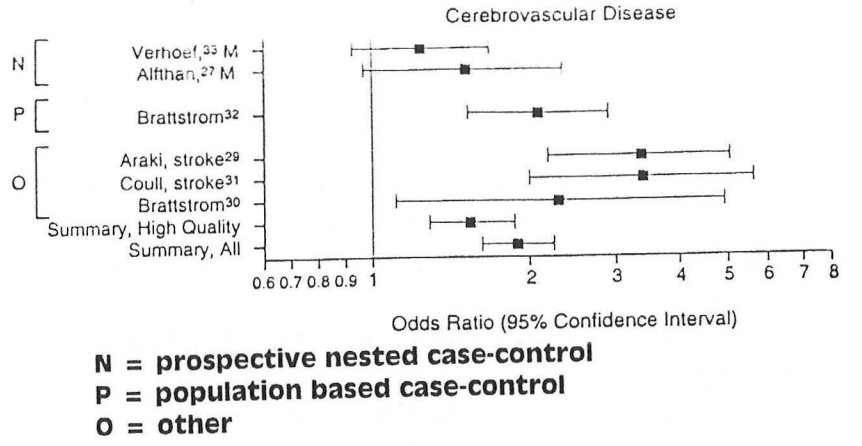


Fig. 19. Odds ratio of cerebrovascular disease if increase plasma level of homocysteine by 5 μmol/L.

Peripheral vascular disease (PVD)

The second most frequent site of vascular events in patients with CBS deficiency is in the peripheral vasculature and the strongest relationship with hyperhomocystinemia is in this subset of patients. Again, the first studies showing a relationship between plasma levels of homocysteine and PVD employed the methionine loading test. In one such study seven of 25 patients under the age of 50, without hypertension, diabetes or hyperlipidemia, who had PVD proven by angiography had elevated (i.e. > 2 S.D.) non-protein bound plasma homocysteine levels after methionine loading (Boer *et al.* 1985).

A total of two population-based, and seven case-control studies, have been performed to examine the relationship between plasma homocysteine levels and peripheral vascular disease (PVD) (for review, see Boushey *et al.* 1995). In only three of these studies was sufficient information provided to calculate the odds ratio, which were all much higher than had been seen in the CVD studies. The overall odds ratio for PVD was 6.8 (CI 2.9 to 15.8) in those studies in which fasting plasma homocysteine levels were measured (Fig. 20).

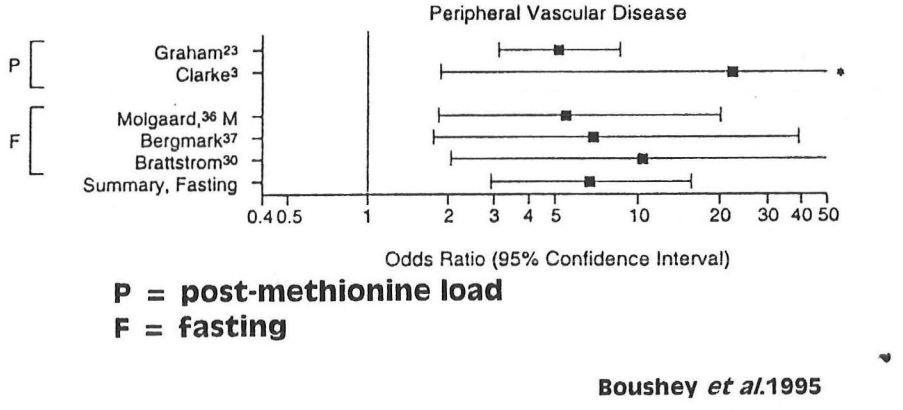


Fig. 20. Odds ratio for PVD associated with elevated plasma homocysteine levels.

Despite the very strong relationship between plasma homocysteine levels and PVD in these studies, there have been some reports, including a well-controlled study from this institution (Valentine *et al.* 1996), that have failed to find an increase in plasma homocysteine levels in patients with early PVD. The reason for the discrepancies between the results of these studies is not clear but may relate to the lack of use of appropriate controls in some studies.

Coronary artery disease (CAD)

Although Boers found a strong relationship between total soluble plasma homocysteine levels (after a methionine load test) and premature CVD and PVD, he found no such relationship with early-onset CAD (Boer *et al.* 1985). However, in subsequent studies in which total plasma homocysteine levels have been measured, CAD patients have repeatedly had ~30% higher levels than controls (for review see Ueland *et al.* 1992; Boushey *et al.* 1995).

Recently Boushey *et al.* (1995) performed a meta-analysis study to examine the link between hyperhomocystinemia and coronary artery disease (CAD), either fatal or nonfatal MI or angiographically-confirmed CAD. A total of 17 studies have been performed and in 14 there was a statistically significant, positive association between plasma homocysteine levels and heart disease. (Fig. 21). Based on this analysis, it was estimated that a high plasma homocysteine level was associated with an odds ratio of 1.7 (CI 1.5 to 1.9) for CAD. When only the studies that used fasting homocysteine levels were included (n=9), the odds ratio was 1.8 (C.I. of 1.6 to 2.0). The effect of a 5 μmol/L increase in plasma homocysteine on the odds ratio CAD was 1.6 (C.I. of 1.4 to 1.7) for men and 1.8 for women.

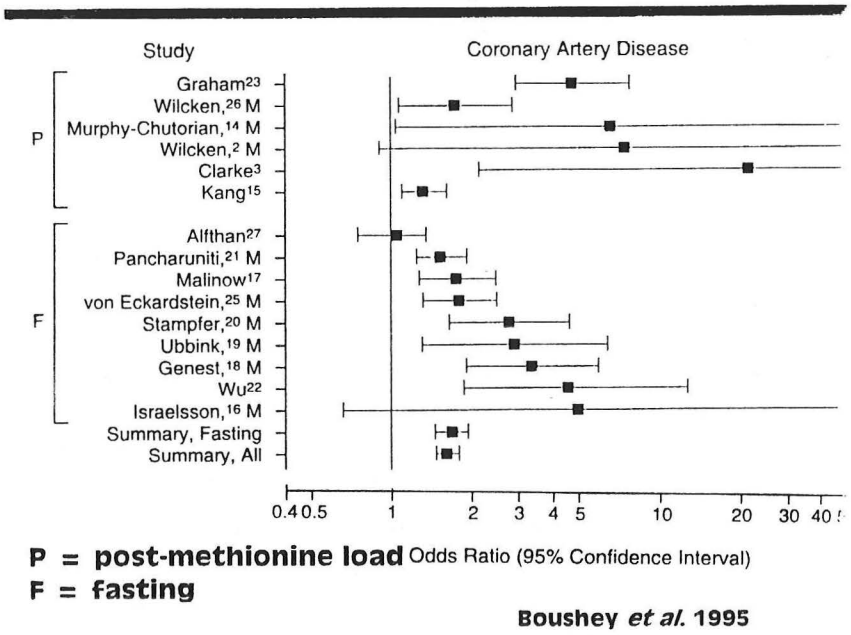


Fig. 21. Odds ratio for CAD associated with elevated plasma homocysteine levels.

None of these studies address the question as to whether the higher plasma homocysteine levels are a cause, or an effect, of CAD. The first prospective study to examine this relationship was the Physicians Health Study (Stampfer *et al.* 1992). This study was a nested, case-control study that was designed to examine the effect of β-carotene and aspirin in male physicians. A total of 14,916 male physicians (ages 40-84) with no history of myocardial infarction (MI) or stroke were enrolled in the study and analysis was performed after a 5 years follow-up. The 271 men who had an MI during the 5 year interval had modestly higher mean baseline homocysteine levels than did their age and smoking-matched controls (11.1 +/- 4.0 μmol/L vs 10.5 +/- 2.8 μmol/L; p=.026) (Fig. 22). A significantly higher number and proportion of cases (n=31, 11%) than controls (n=13, 6%) had plasma levels of homocysteine greater than the 95 percentile when compared to the control group. The individuals with plasma homocysteine levels over the 95th percentile (>15.8 μmol/L) had a 3-fold increased risk of cardiovascular disease when compared to the individuals with levels under the 90th percentile. Plasma levels of homocysteine that were only 12% above the upper limit of normal (for example, men with a plasma level of homocysteine of 15.8 vs. 14.1 μmol/L) were associated with a 3.4 fold increase in risk (C.I. 1.3-8.8). Adjustments for the other risk factors using multivariant analysis did not alter the results of the analysis. This study provided the much needed high plasma levels of homocysteine not secondary to the development of symptomatic vascular disease.

	Homocysteine (nmol/mL)	Homocysteine > 95th	Homocysteine < 95th
Cases	11.1 ± 4.0*	31	231
Control	10.5 ± 2.8	13	244

*P = .026; Relative risk = 3.13.

Fig 22. Plasma homocysteine levels Physicians Health Study.

The only other prospective study also showed a positive association between plasma homocysteine levels and heart disease is the Tromo Study (Arnesen *et al.* 1995).

To determine if the thermolabile form of MTHFR may be responsible for elevated plasma homocysteine levels in some of the individuals with hyperhomocystinemia and CAD, 212 individuals with greater than 70 % occlusion of a major vessel, or greater than 50% narrowing of the left main coronary artery were screened for the presence of thermolabile MTHFR using lymphocyte extracts. A total of 18.1% of those with CAD had the thermolabile defect, in contrast to 7.9% of those with no vascular lesions (Kang *et al.* 1993) (Fig. 23). Importantly, there was no statistically significant difference in the plasma levels of homocysteine between the groups (Fig. 24), although within each group, the MTHFR homozygotes had significantly higher plasma levels of homocysteine.

Fig. 23.

Thermolabile Methylenetetrahydrofolate Reductase In Control Patients and Patients With Coronary Artery Stenosis

	Severe Stenosis, Group 1	Mild to Moderate Stenosis, Group 2	No Stenosis*, Group 3
No. with thermolabile methylenetetrahydrofolate reductase	28	11	8
No. in group	155	83	101
Percentage with thermolabile methylenetetrahydrofolate reductase†	18.1†	13.4†	7.9†

Kang, Circulation, 1993

Fig. 24.

Homocyst(e)ine, Folic Acid, and Cyanocobalamin Levels in Patients With Thermolabile Methylenetetrahydrofolate Reductase

Variable	Severe Stenosis, Group 1	Mild to Moderate Stenosis, Group 2	No Stenosis, Group 3
No. of patients	27	9	8
Homocyst(e)ine, nmol/mL	14.86±5.85	15.36±5.70	13.39±3.80
Folic acid, ng/mL	8.02±7.02	8.65±8.17	10.10±8.55
Cyanocobalamin, pg/mL	433.5±282.9	694.9±521.1	407.0±159.9

No significant differences ($P > .05$) among groups 1, 2, and 3.

Kang, Circulation, 1993

In more recent studies, the defect in the MTHFR gene itself has been analyzed in patients with non-coronary as well as coronary vascular disease. Homozygosity for the MTHFR thermolabile allele was 2-5 times more frequent in the cardiovascular disease group than in the control group (Kluijmans *et al.* 1995, Enghersen *et al.* 1995). In the populations that have been studied, the frequency of homozygotes in the control group is ~ 5% and the frequency in the disease groups ranges from 15 to 28%. In the study of Kluijmans *et al* (1995), the risk ratio for having cardiovascular disease in homozygous for the thermolabile allele was 3.1 (C.I. 1.0-9.2) (Fig. 25), despite these individuals having only a modestly increased plasma homocysteine level (Fig. 26).

Fig. 25. **MTHFR Geneotype Distribution amount Cardiovascular Disease Patients and Control Groups**

Genotype	Cardiovascular Disease Patients	Controls
+ / +	15% ($n = 9$)	5.4% ($n = 6$)
+ / -	25% ($n = 21$)	37.8% ($n = 42$)
- / -	50% ($n = 30$)	56.8% ($n = 63$)

Kluijmans *et al.* 1995

Fig. 26.

Relationship between Fasting and Post-Methionine-Loading Plasma Homocysteine Concentrations and MTHFR Genotype

	+/+ (*n* = 15)	+/- (*n* = 61)	-/- (*n* = 93)
Fasting homocysteine[a] (μmol/liter)	16.3 ± 8.3	13.4 ± 4.0	12.3 ± 3.6
Post-methionine-loading homocysteine[b] (μmol/liter)	49.8 ± 20.0	41.9 ± 18.0	38.4 ± 11.7

Kluijmans *et al.* 1995

Thus, it appears that there is an increase in for homozygosity of the thermolabile form of MTHFR among patients with vascular disease. A number of questions remain to be answered about this association. Is the increase in risk associated with the MTHFR defect due to the secondary increase in homocysteine in cells, or in plasma? The back-up system for methylation of homocysteine to methionine (the betaine-homocysteine methyltransferase) is not operative in nonhepatic tissues and because of this endothelial and vascular smooth muscle cells may be more sensitive to increases in intracellular homocysteine. Better methods need to be developed to assess the metabolic consequences of mild MTHFR deficiency in cells.

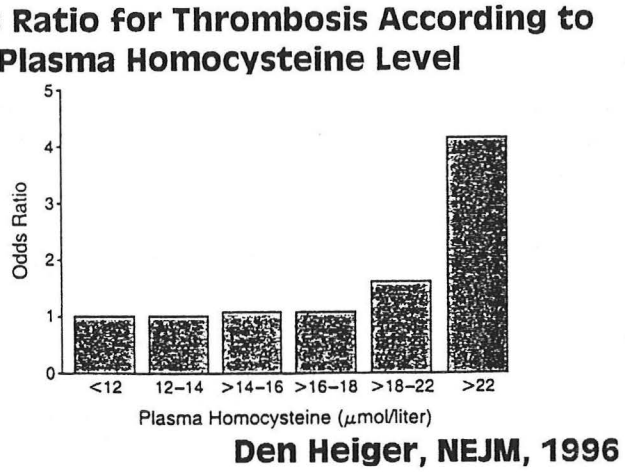
Venous thrombosis

The venous circulation is the most frequent site of symptomatic thrombosis in subjects with homocystinuria (Mudd *et al.* 1981). In light of this, it is surprising how few studies have been done to determine if hyperhomocystinemia predisposed to venous thrombosis and pulmonary embolism. In most studies, patients with venous thrombosis tended to have higher plasma levels of homocysteine than normal controls. In a sample of 185 patients with recurrent DVT, 25% had fasting plasma homocysteine levels greater than the 90th percentile (18.6 μmol/L) vs 9% of the controls (Den Heijers *et al.* 1995). In this study, the odds ratio for having venous thrombosis if the plasma homocysteine level was greater than the 90th percentile was 2 (CI 1.5-2.7) after adjustment for age, sex and menopausal status.

Evidence for a graded response associated with plasma levels of homocysteine was found in another study by den Heijer *et al.* (1996) that measured plasma homocysteine levels after the first episode of DVT (n = 269; mean age, 44 years) (Fig. 27). Though the mean level of homocysteine was only modestly elevated in the DVT group (12.9 μmol/L vs. 12.3 μmol/L in controls), twice the number of patients (10%) as controls (5%) had plasma levels of homocysteine greater than the 95th percentile (i.e. >18.5 μmol/L). Exclusion of subjects with other genetic defects associated with thrombophilia (i.e., protein C or protein S deficiency, anti-thrombin III deficiency, or factor V-Leiden), which do not correlate with high plasma levels of homocysteine (Bienvenu *et al.* 1993), did not change the odds ratio in this study. However, the risk for having a thrombotic event associated with hyperhomocystinemia is significantly less than for factor V-Leiden (odds ratio 2.2 vs 9.5) (Den

Heijer *et al.* 1996).

Fig. 27



Thus, there is strong evidence linking the presence of high plasma levels of homocysteine with vascular disease, especially peripheral vascular disease, though the degree of elevation is much less than seen in any of the genetic disorders associated with severe hyperhomocystinemia, such as CBS deficiency, severe MTHFR deficiency or rare defects in B_{12} metabolism. Though additional studies need to be performed, it appears that heterozygosity for a CBS gene defect is not a frequent cause of hyperhomocystinemia in patients with vascular disease. In contract, homozygosity for the thermolabile form of MTHFR does appear to be associated with CAD, but more studies are needed to confirm these findings and determine if the relationship holds true for subjects with CVD, PVD and venous thrombosis.

There is no evidence that the association between homocystinemia and vascular disease is due to a secondary elevation in plasma homocysteine levels caused by another cardiac risk factor. Specifically, there is no correlation between plasma homocysteine levels and blood pressure levels, plasma lipoprotein levels, smoking or diabetes. What is the mechanism responsible for the elevated plasma levels, of homocysteine in subjects with vascular disease? In most studies, the frequency distribution of homocysteine levels appears to be slightly shifted to the right in the vascular disease group, which is most consistent with the increase in homocysteine level being multifactorial in nature, rather than due to the effect of a single major gene.

DETERMINANTS OF PLASMA HOMOCYSTEINE LEVELS

Family studies have demonstrated that environmental as well as genetic factors contribute to fasting plasma levels of homocysteine. There is a high concordance of plasma homocysteine levels in identical twins and sibling pairs, as would be expected for a trait that is genetically determined (Berg *et al.* 1992; Reed *et al.* 1991, Williams, *et al.* 1990).

The factors known to alter plasma levels of homocysteine are listed in Table I. They have been classified into two groups- non-genetic and genetic. The only factors listed that are associated with lower, rather than higher, plasma homocysteine level are Down Syndrome, pregnancy and tamoxifen (Kang *et al.* 1986). Recall that CBS is located on chromosome 21 and so individuals with Down Syndrome have three copies of the enzyme. Associated with this extra copy of the enzyme is an increase in CBS activity and a significantly lower plasma homocysteine levels (Chadefaux *et al.* 1985). Individuals with Down syndrome may have a decrease in atherosclerosis, although the numbers of affected individuals carefully examined for vascular lesions has been small (Murdoch *et al.* 1977).

TABLE 1. **FACTORS THAT MODIFY PLASMA LEVELS OF HOMOCYSTEINE**

NON-GENETIC	GENETIC
1. Vitamin B_{12} deficiency	1. CBS deficiency
2. Folic acid deficiency	2. 5,10-MTHFR deficiency
3. Vitamin B_6 deficiency	Thermolabile form of MTHFR
4. Nitrous oxide	3. Defects in B_{12} absorption, transport and metabolism
5. Methotrexate	Immerslund syndrome
6. Phenytoin	Transcobalamin II deficiency
7. Azaribine (6-azauridine)	Cobalamin defects (*cblC-F*)
8. Bile acid sequestrants / niacin	4. Down syndrome
9. Tamoxifen	
10. Psoriasis	
11. Renal insufficiency	
12. Cardiac transplantation	
13. Pregnancy	

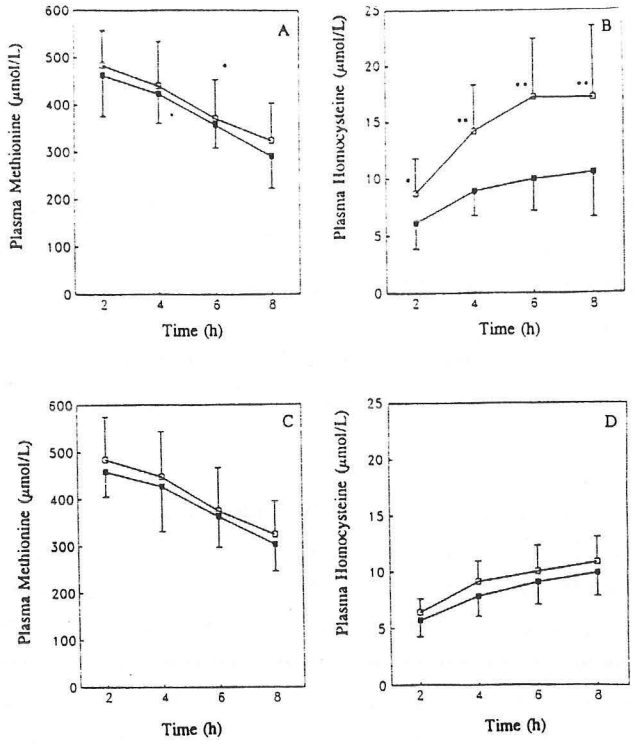
Dietary deficiencies of folate, vitamin B_{12} and B_6, which are cofactors or substrates in the homocysteine metabolic pathway, are the most important environmental causes of elevated plasma homocysteine levels, and will be discussed independently in the next section. Nitrous oxide administration is associated with a significant increase in the plasma level of homocysteine, presumably due to oxidation and inactivation of cobalamin. Plasma levels of homocysteine increase after methotrexate administration; the reduction in dihydrofolate reductase activity limits the supply of 5-methyltetrahydrofolate available for the methylation of homocysteine. Interestingly, subjects given methotrexate also have an increased incidence of thrombosis. Patients with psoriasis have hyperhomocystinemia, perhaps due to cellular hyperproliferation and secondary folate deficiency (Refsum *et al.* 1989). Azaribine (6-azauridine triacetate) is a vitamin B_6 antagonist that was used to treat psoriasis but the drug was taken off the market due to the high frequency of thromboembolism associated with its administration. Participants in the Cholesterol-Lowering Atherosclerosis study (CLAS) trial that took bile acid sequestrants and niacin were noted to have significantly higher

plasma levels of homocysteine that placebo controls (15.8 μmol/L vs 10.6 μmol/L) (Blankenhorn *et al.* 1991). The mechanism responsible for this effects is not known. Bile acid sequestrants may interfere with folate absorption and nicotinamide (and, thus, perhaps niacin), undergoes methylation during its catabolism, limiting the availability of methyl groups to convert homocysteine to methionine. Treatment of post-menopausal women with tamoxifen, an estrogen antagonist was associated with a ~25% drop in plasma homocysteine. It has been suggested that this reduction may contribute to the reduced incidence of cardiovascular events associated with tamoxifen therapy (Anker *et al.* 1995).

Individuals with chronic renal insufficiency have plasma levels of homocysteine that are 2-4 fold higher than normal (mean levels of plasma homocysteine of 22.7 μmol in patients vs. 9.5 μmol in controls) (Hultbert *et al.* 1993; Bostrom *et al.* 1995). About 75% of the homocysteine formed is linked to the production of creatine and creatinine, and this may contribute to the correlation between plasma levels of homocysteine and creatinine seen in some studies. But presumably the major cause of the increase in plasma homocysteine levels in patients is due to decreased excretion. Hemodialysis is associated with only a 30% decrease in the plasma homocysteine level since most of plasma homocysteine is protein bound. The plasma level of homocysteine falls significantly with high-dose folate treatment, even in renal failure patients with normal plasma levels of folate (Wilcken *et al.* 1988) (see below).

Plasma levels of homocysteine increase ~ 70% after cardiac transplantation (Berger *et al.* 1995). The reason for the elevation of plasma homocysteine levels in these subjects is not known but cannot be attributed solely to the associated reductions in renal function (Ambrosi *et al.* 1994). It may be due to the effect of immunosuppressive drugs and secondary folate deficiency. The relationship between the plasma homocysteine levels and the coronary artery vasculopathy, which is the primary cause of late mortality in transplant patients, is not known. In one small study, the severity of coronary vascular lesions did not correlate with plasma homocysteine levels (Ambrosi *et al.* 1994).

Plasma levels of homocysteine are lower in South African Blacks than in Caucasians, especially after methionine challenge (11 vs. 18 μmol) (Ubbink, *et al.* 1995) (Fig. 28). The differences are not due to differences in methionine absorption since the plasma methionine levels post oral challenge were identical. Nor were there significant differences in plasma folate levels between the groups. Blacks have lower plasma pyridoxal 5′ -phosphate levels, which may be due to blacks having lower levels of pyridoxal kinase. This would, however, be expected to cause an increase in post-methionine homocysteine levels. The mechanism for the observed inter-ethnic differences in plasma homocysteine levels is not known, but it is interesting that blacks, especially those in Africa, have a reduced incidence of vascular disease.



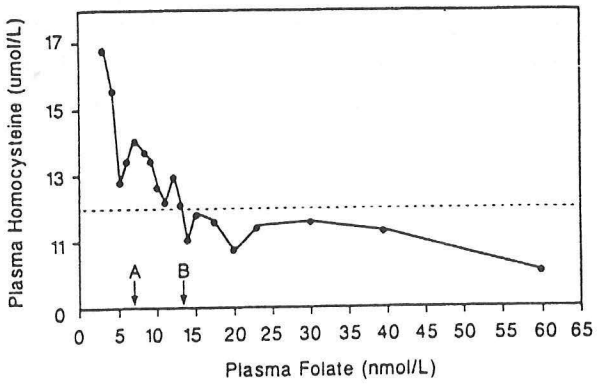
Ubbink, Am. J. Clin. Nutr., 1995

Fig. 28. Mean plasma methionine and honocysteine concentrations after methionine loading in black (n = 12; ■) and white (n = 18; □) men before (top) and after (bottom) 6 weeks of vitamin supplementation.

VITAMIN DEFICIENCIES AND HYPERHOMOCYSTINEMIA

As suggested previously, deficiencies in the two vitamins that serve as cofactors for enzymes in the homocysteine metabolic pathway, B_6 and B_{12}, and deficiencies in folate, which is a substrate for methionine synthesis, are important contributors to hyperhomocystinemia in the population. The frequency of deficiencies in one of these three vitamins is remarkably high in the U.S. population, especially among the elderly. Selub *et al.* (1993) took detailed dietary histories and measured the nonfasting plasma levels of folate, vitamin B_{12} , vitamin B_6 and homocysteine in 1160 participants in the Framingham Heart Study, a population-based cohort of elderly (age 67-97) people. Almost one-third of the sample had a plasma homocysteine level over 14 μmol/L (> 90th percentile) and in two-thirds of these there was evidence of an associated vitamin deficiency. There was an inverse nonlinear association between plasma homocysteine levels and the plasma levels and dietary intake of both folate and vitamin B_6 as well as with the plasma level of vitamin B_{12} (Fig. 29).

Fig. 29



A - NHANES II
B - WHO

Lewis *et al.* 1992

The strongest relationships were observed between the plasma homocysteine level and the estimated intake and plasma level of folate. Approximately 20% of the subjects who had a plasma homocysteine level >15.8 μmol/liter consumed less than the recommended daily intake of folate, which is 200 ug/day for men, and 180 ug/day for women. An additional 20% of the Framingham sample consumed 200-400 μg/day, and they also had significantly higher plasma homocysteine levels. Only after consumption of 400 μg/day of folate (which, ironically used to be the RDA) and a serum folate of 15 nmol/L, did the plasma homocysteine levels plateau. Shown in Figure 30 is the plasma homocysteine level at which NHANES II and the WHO had set the lower limit of normal plasma folate (6.7 nmol vs 13.6 nmol/L) (Fig. 30).

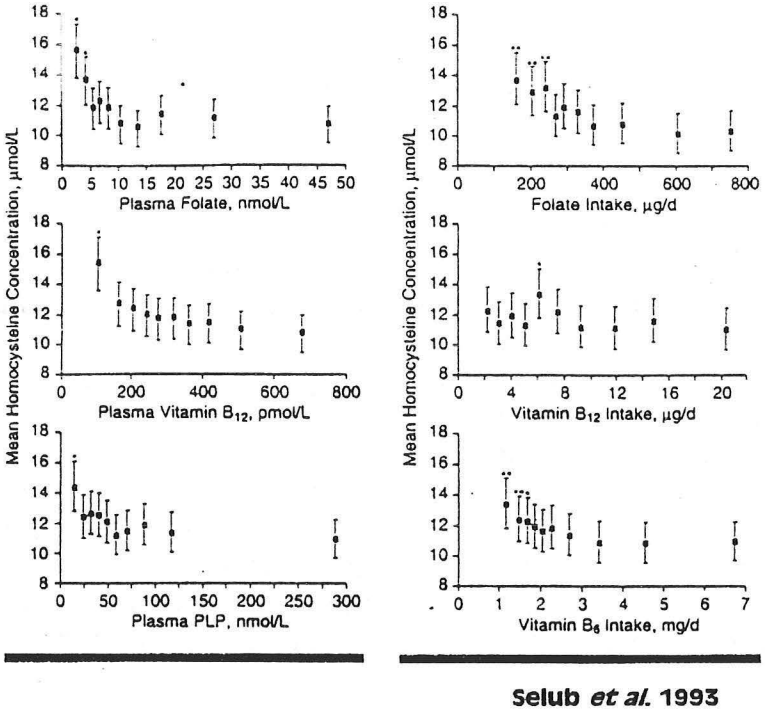
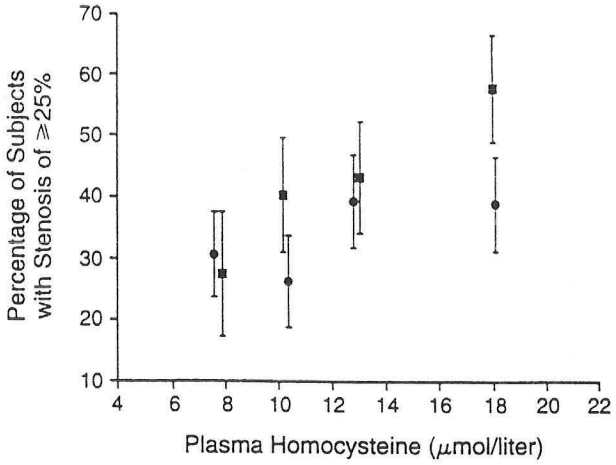


Fig. 30. Relationship between plasma folate, B_{12} and B_6 levels (left) and intake (right) and plasma homocysteine.

Selub went on to show in this same population that levels of plasma homocysteine within the so-called "normal range" were associated with an increase in vascular disease. Selub measured nonfasting plasma levels of homocysteine, folate , B_{12} and B_6 and the amount of carotid stenosis in 1041 subjects from the Framingham study using carotid ultrasonography (Selub *et al.* 1995). There was a direct relationship between plasma homocysteine level and the percentage of subjects with significant (i.e. > 25%) carotid stenosis (Fig.31). The amount of stenotic narrowing was inversely related to the plasma folate and B_6 levels and to the folate intake, and the effect was independent of other cardiovascular risk factors. The individuals with plasma homocysteine levels in the top quartile (>11.4 μmol/L) had an odds ratio of 2.1 for having significant lesions. Thus, individuals with so-called "high normal" plasma homocysteine levels were at increased risk for vascular disease.

Association Between Plasma Homocysteine Levels and Extracranial-Artery Stenosis

Fig. 31.



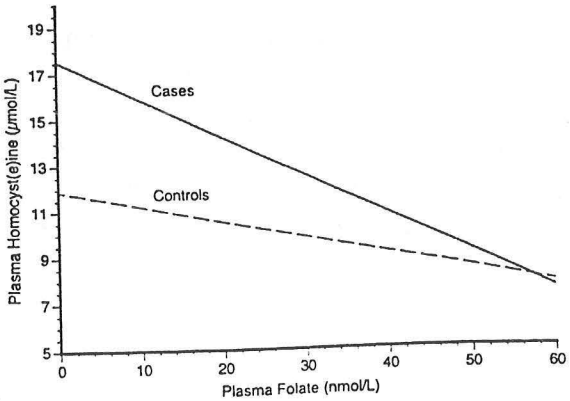
Age-Adjusted Prevalence of Maximal Extracranial Carotid-Artery Stenosis of ≥25 Percent in Men (■) and Women (●), According to the Quartile of Plasma Homocysteine Concentration.

Selub *et al.* 1995

It is interesting that in two studies (Brattsom *et al.* 1992 and Hopkins *et al.* 1995) the slope of the inverse relationship between plasma folate and homocysteine levels was steeper in cases than in the control group (Fig. 32). This may suggest a shared underlying genetic difference in the vascular disease group that predisposes these individuals to higher plasma homocysteine levels for any given level of folate. The common thermolabile MTHFR polymorphism is the most likely known genetic defect that contributes to this observation, but unfortunately was not assayed in either of these studies. It may be that the animal protein-rich diet consumed in industrialized societies, which is very high in methionine, requires a higher intake of folate. Individuals with a sluggish MTHFR may be particularly susceptible to diets rich in methionine and low in folate. Though it has not been conclusively shown that individuals with the thermolabile MTHFR gene defect have a greater sensitivity to folate insufficiency, folate supplementation dramatically decreased the level of plasma homocysteine in at least two individuals with the thermolabile form of MTHFR (Kang *et al.*1988).

Fig. 32

Relationship Between Plasma Folate and Homocysteine Levels in Individuals with Premature Coronary Artery and Age Matched Controls



Hopkins *et al.* 1995

Despite the inverse relationship between plasma homocysteine levels and plasma B_{12}, B_6 and folate levels, folate supplementation alone is as effective as combination vitamin therapy (i.e. folate, vitamin B_6 and vitamin B_{12}) in lowering plasma homocysteine levels (Ubbink *et al.* 1994). B_{12} is effective in lowering plasma homocysteine levels only if there is a deficiency of B_{12}.. Vitamin B_6 supplementation may reduce peak plasma homocysteine levels after a methionine load, or a protein meal, but does not usually significantly affect fasting levels of homocysteine. At least 9 intervention studies have shown significant decreases in plasma homocysteine levels with 650 to 10,000 μg of folic acid supplementation, even in subjects with normal plasma level of folate, B_{12} and B_6 (Boushey *et al.* 1995, Wicken *et al.* 1988). The magnitude of reduction in plasma homocysteine levels is proportional to the starting homocysteine level, and a plateau of homocysteine-lowering effect is reached at a folate intake of ~ 400 μg.

Unfortunately, no studies have been performed to determine if folate supplementation is associated with a decrease in vascular disease. Such studies are needed to test whether lowering plasma homocysteine levels with folate administration will influence the development of vascular disease.

Three major population-based strategies that have been proposed to correct the high frequency of folate deficiency and secondary hyperhomocystinemia in our population and the predicted effect of these three different strategies on cardiac deaths is shown in Fig. 33.

1) Educational programs to promote an increase in dietary folate intake.
2) Encourage folate supplementation as part of a multivitamin.
3) Fortify flour with folate.

Fig. 33. **Potential Impact on Prevention of Deaths From Coronary Heart Disease (CHD) for Men and Women Aged 45 Years and Older Based on Three Intervention Strategies**

Strategy	Increase in Folic Acid, μg/d*	Percentage of CHD Deaths Potentially Prevented		Annual No. Of Potentially Preventable Deaths†	
		Male	Female	Male	Female
Nutriton education					
40% effective	50‡	2.2	1.7	7500	6000
80% effective	50‡	4.3	3.3	15000	11500
Folic acid supplements					
25% effective	400	2.2	1.8	12500	6500
50% effective	400	4.4	3.36	15500	12500
Food fortification of flour and cereal products, μg/g					
140/100	170 M 160 F	6.6	4.8	23000	17000
350/100	350 M	8.8	5.4	30500	19000

Though the first two strategies would be expected to be effective, they would rely entirely on the compliance of asymptomatic individuals. To consume a sufficient amount of folate to maximally lower plasma homocysteine levels (400 μg) would require ingestion of at least 5 servings of fruits

and vegetables a day. The addition of folate as a vitamin supplement has all the problems associated with motivating individuals to take pills.

The third strategy, fortifying flour (140-350 μg of folate/100 g), has been approved by the U.S. Food and Drug Administration and is now in a bill before Congress. Fortification of flour with folate would be estimated to result in a 3-5 μmol/L fall in plasma homocysteine levels and a 5-10% fall in cardiac deaths (Boushey *et al.* 1995). It would also be expect to decrease the incidence of neural tube defects by ~ 50%, another adverse clinical outcome associated with folate deficiency. A potential problems with folate fortification of flour is that it may mask the hematological manifestation of B_{12} deficiency while patients develop severe, and oftentimes irreversible, neurological symptoms. But this reportedly happens very rarely and only when very high doses of folic acid (5000 mg) are given to B_{12} -deficient individuals. To address this potential problem B_{12} may be included in the supplementation as well (Boushey *et al.* 1995).

CONCLUSIONS

The fact that several different genetic defects causing elevated plasma homocysteine levels are associated with premature vascular disease is the most compelling evidence that homocysteine itself is responsible for the vasculopathy. Surprisingly little progress has been made regarding the pathogenic link between homocysteine and vascular disease. A problem with all studies performed to date is that they presuppose that addition of various forms of homocysteine to the circulation *in vivo,* or to cells *in vitro,* has the same effect as high intracellular concentration of homocysteine. Hyperhomocystinemia may be a consequence rather than the cause of metabolic disturbance responsible for the vasculopathy. Metabolic defects in the homocysteine-methionine pathway may have important yet-to-be-defined adverse effects on the metabolism of the cell. The development of animal models and cell lines with molecular defects in the various enzymes in the homocysteine metabolic pathway will allow more detailed dissection of the intracellular effects of the various genetic defects.

Despite the lack of direct evidence that homocystinemia causes vascular disease, the consistency of the findings of moderately elevated plasma levels of homocysteine in individuals with both arterial and venous disease suggests a causal relationship. Proving such a relationship will require the performance of additional large prospective studies to examine the effect of folate supplementation, and the associated fall in homocysteine, on the incidence of vascular events.

Accepting the limitations of the available information, should we be measuring plasma levels of homocysteine in our patients, and should we be treating hyperhomocysteinemic patients with folate? Routine measurement of plasma homocysteine does not seem warranted at this time. The assays for homocysteine are not standardized and the differences between normal and risk-associated levels of plasma homocysteine are narrow and not clearly defined. Given all the available information, active encouragement to increase the intake of fruits and vegetables in the population is warranted, not only as a strategy to possibly reduce the development vascular disease, but other diseases as well. Since folate is well tolerated and safe (except in individuals with vitamin B_{12} deficiency), the threshold

to prescribe folate should be low in individuals with vascular disease, or multiple risk factors for vascular disease.

One milligram of folate a day may help keep the doctor away!

ACKNOWLEDGMENT

I wish to thank Laura Fuller for her excellent assistance in the preparation of the protocol and slides.

Bibliography

Ambrosi, P., A. Barlatier, G. Habib, D. Garcon, B. Kreitman, P.H. Roland, S. Saingra, D. Metras, and R. Luccioni. 1994. Hyperhomocysteinaemia in heart transplant recipients. *Eur. Heart J.* 15:191-1195.

Anker, G., P.E. Lønning, P.M. Ueland, H. Refsum, and E.A. Lien. 1995. Plasma levels of the atherogenic amino acid homocysteine in post-menopausal women with breast cancer treated with tamoxifen. *Int. J.Cancer*. 60:365-368.

Barber, G.W., and G. L. Spaeth. 1967. Pyridoxine therapy in homocystinuria. *Lancet*. 1:337.

Berg, K. , M.R. Malinow, P. Kierulf, and B. Upson. 1992. Population variation and genetics of plasma homocyst(e)ine level. *Clin. Genet*. 41:315-321.

Berger, P.B., J. D. Jones, L.J. Olson, B.S. Edwards, R.P. Frantz, R.J. Rodeheffer, B.A. Kottke, R.C. Daly, and C.G.A. McGregor. 1995. Increase in total plasma homocysteine concentration after cardiac transplantation. *Mayo Clin. Proc*. 70:125-131.

Bienvenu, T., A. Ankri, B. Chadefaux, G. Montalescot and P. Kamoun. 1993. Elevated total plasma homocysteine, a risk factor for thrombosis. Relation to coagulation and fibrinolytic Parameters. *Thrombosis Res*. 70:123-129.

Blankenhorn, D.H., M.R. Malinow, and W.J. Mack. 1991. Colestipol plus niacin therapy elevates plasma homocyst(e)ine levels. *Coronary Artery Dis*. 2:357-360.

Blom, H.J., D.P.E. Engelen, G.H.J. Boers, A.M. Stadhouders R.C.A. Sengers, R. De Abreu, M.T.W.B. TePoele-Pothoff, and J.M.F. Trijbels. 1992. Lipid peroxidation in homocystinemia. *J. Inherited Metab. Dis*. 15:419.

Blom, H.J., and E.F. van der Molen. 1994. Pathobiochemical implications of hyperhomocystinemia. *Fibrinolysis*. 8:(Suppl. 2), 86-87.

Boers, G.H.J., A.G.H. Smals, F.J.M. Trijbels, B. Fowler, J.A.J.M. Bakkeren, H.C. Schoonderwaldt, W.J. Kleijer, and P.W.C. Kloppenborg. 1985. Heterozygosity for homocystinuria In premature peripheral and cerebal occlusive arterial disease. *N. Engl. J. Med.* 313:(12) 709-715.

Bostom, A.G., D. Shemin, K.L. Lapane, J.W. Miller, P. Sutherland, M. Nadeau, E. Seyoum, W. Hartman, R. Prior, P.W.F. Wilson, and J. Selhub. 1995. Hyperhomocystinemia and traditional cardiovascular disease risk factors in end-stage renal disease patients on dialysis: a case-control study. *Atherosclerosis*. 114:93-103.

Boushey, C.J., S.A.A. Beresford, G.S. Omenn, and A.G. Motulsky, 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *JAMA* 274:(13) 1049-1057.

Brattström, Lars, B. Israelsson, and B. Hultberg. 1989. Plasma homocysteine and methionine tolerance in early-onset vascular disease. *Haemostasis* 19:(suppl 1) 35-44.

Brattström, L., B. Israelsson, B. Norrving, D. Bergqvist, J. Thörne, B. Hultberg, and A. Hamfelt. 1990. Impaired homocysteine metabolism in early-onset cerebral and peripheral occlusive arterial disease. Effects of pyridoxine and foic acid treatment. *Atherosclerosis*. 81:51.

Brattström, L., A. Lindgren, B. Israelsson, M.R. Malinow, B. Norrving, B. Upson, and A. Hamfelt. 1992. Hyperhomocysteinaemia in stroke: prevalence, cause, and relationships to type

of stroke and stroke risk factors. *Eur. J. Clin. Invest.* 22:214-221.
Caron, N.A.J., and D.W. Neill. 1962. Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland. *Arch. Disease Childhood.* 37:505-513.
Chadefaux, B., M.O. Rethore, and O. Raoul. 1985. Cystathionine beta synthase: gene dosage effect of trisomy 21. *Biochem. Biophys. Res. Commun.* 128:40-44.
Clarke R. 1990. The Irish experience. In: Robinson K, ed. Homocysteinaemia and vascular disease. Luxembourg: Commission of the European Communities. 41-48.
Clarke, R., L. Daly, K. Robinson, E. Naughten, S. Cahalane, B. Fowler, and Ian Graham. 1991. Hyperhomocysteinemia: an independent risk factor for vascular disease. *N. Engl. J. Med.* 324: (17) 1149-1155.
Cox, R.P. 1991. Medical genetics for internists homocystinuria and marfan syndrome. pp1-28.
Dahlback B. 1987. Inherited thrombophilia: resistance to activated protein C as a pathogenic factor of venous thromboembolism. *Blood.* 85:607-614.
De Groot, P.G., C. Willems, G.H.J. boers, M.D. Gonsalves, W.G. Van Aken, and J.A. Van Mourik. 1983. Endothelial cell dysfunction in homocystinuria. *Eur. J. Clin. Invest.* 13:405-410.
den Heijer, M., H. J. Bloom, W.B.J. Gerritis, F.R. Rosendaal, H.L. Haak, P.W. Wijermans, and G.M.J. Bos. 1995. *Lancet.* 345:882-885.
den Heijer, M., T. Koster, H.J. Blom, G.M.J. Bos, E. Briët, P.H. Reistma, J.P. Vanderbroucke, and F.R. Rosendaal. 1996: Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. *N. Engl. J. Med.* 334:(12) 759-762
Engbersen, A.M.T., D.G. Franken, G.H.J. Boers, E.M.B. Stevens, F.J.M. Trijbels, and H.J. Blom. 1995. Thermolabile 5,10-methylenetetrahydrofolate reductase as a cause of mild hyperhomocystinemia. *Am. J. Hum. Genet.* 56:142-150.
Fryer, R.H., B.D. Wilson, D.B. Gubler, L.A. Fitzgerald, and G.M. Rodgers. 1993. Homocysteine, a risk factor for premature vascular disease and thrombosis, induces tissues factor activity in endothelial cells. *Arterioscler. Thromb.* 13:1327-1333.
Gallagher, P.M., P. Ward, S. Tan, E. Naughten, J.P. Kraus, G.C. Sellar, D.J. McConnell, I. Graham, and A.S. Whitehead. 1995. High frequency (71%) of cystathionine beta-synthase mutation G307S in Irish homocystinuria patients. *Human Mutation.* 6:177-180.
Goldstein, J.L., B.K. Campbell, and S.M. Gartler. 1973. Homocystinuria: Heterozygote detection using phytohemagglutinin-stimulated lymphocytes. J. Clin. Invest. 52:218.
Goyette, P., P. Frosst, S. D.S. Rosenblatt and R. Rozen, 1995. Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. *Am. J. Hum. Genet.* 56:1052-1059.
Goyette, P., J.S. Sumner, R. Milos, A.M.V. Duncan, D.S. Rosenblatt, R.G. Matthews and R. Rozen, 1994. Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification. *Nature Genetics* 7:195-200.
Hajjar, K.A. 1993. Homocysteine-induced modulation of tissue plasminogen activator binding to its endothelial cell membrane receptor. *J. Clin. Invest.* 91:2873-2879.
Harker, L.A., S.J. Slichter, C.R. Scott, and R. Ross, 1974. Homocystinemia. *N. Engl. J. Med.* 291:(11) 537-543.
Harpel, P.C., V.T. Chang, and W. Borth. 1992. Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: A potential biochemical linked between

thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proc. Natl. Acad. Sci. USA.* 89:10193-10197.

Heinecke, J.W., H. Rosen, L.A. Suzuki, and A. Chait. 1987. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.* 262: 10098-10103.

Hill-Zobel, R.L., R.E. Pyeritz, U. Scheffel, O. Malpica, S. Engin, E.E. Camargo, M. Abbott, T.R. Guilarte, J. Hill, P.A. McIntyre, *et al.* 1982. Kinetics and distribution of ^{111} indium-labeled platelets in patients with homocystinuria. *Eur. J. Clin. Invest.* 13405-410.

Hopkins, P.N., L.L. Wu, S.C. Hunt, B.C. James, G.M.Vincent and R.R. Williams, 1995. Higher plasma homocyst(e)ine and increased susceptibility to adverse effects of low folate in early familial coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* 15:1314-1320.

Hultberg, B., A. Andersson and G. Sterner, 1993. Plasma homocysteine in renal failure. *Clin. Nephrol.* 40:(4) 230-234.

Kang, S-S., P.W.K. Wong, J. Zhou, and H.Y. Cook. 1986. Preliminary report: total homocyst(e)ine in plasma and amniotic fluid of pregnant women. *Metabolism.* 35:889-891.

Kang, S-S., J. Zhou, P.W.K. Wong, J. Kowalisyn, and G. Strokosch, 1988. Intermediate homocystinemia: a thermolabile variant of methylenetetrahydofolate reductase. *Am J. Hum. Genet.* 43:414-421.

Kang, S-S., E.L. Passen, N. Ruggie, P.W.K. Wong, and H. Sora. 1993. Thermolabile defect of methylenetetrhydrofolate reductase in coronary artery disease. *Circulation.* 88:1463-1469.

Kang, S-S., P.W.K. Wong, H-G. O. Bock, A. Horwitz and A. Grix, 1991. Intermediate Hyperhomocystinemia resulting from compound heterozygosity of methylenetetrahydrofolate reductase mutations. *Am. J. Hum. Genet.* 48:546-551.

Kanwar, Y.S., J.R. Manaligod, and P.W.K. Wong, 1976. Morphologic studies in a patient with homocystinuria due to 5,10-methyleneterahydrofolate reductase deficiency. *Pediatr. Res.* 10: 598-609.

Kluijtmans, L.A.J., H.J. Blom, G.H.J. Boers, B.A. van Oost, F.J.M. Trijbels, L.P.W.J. van den Heuvel. 1995. Two novel missense mutations in the cystathionine β-synthase gene in homocystinuric patients. *Hum. Genet.* 96:249-250.

Kluijtmans, L.A.J., L.P.W. J. Vanden Heuvel, G.H.J. Boers, P. Frosst, E.M.B. Stevens, B.A. van Oost, M. Den Heijer, F.J.M. Trijbels, R. Rozen and H.J. Blom, 1996. Molecular genetic analysis in mild hyperhomocystinemia: A common mutation in the methylenetetrhydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am. J. Hum. Genet.* 58:35-41.

Kraus, J.P., K. Le, M. Swaroop, T. Ohura, T. Tahara, L.E. Rosenberg, M.D. Roger and V. Kožich, 1993. Human cystathionine β-synthase cDNA: sequence, alternative splicing and expression in cultured cells. *Hum. Mol. Genet.* 2:(10) 1633-1638.

Kraus, J.P. 1994. Molecular basis of phenotype expression in homocystinuria. *J. Inher. Metab. Dis.* 17:383-390.

Lentz, S.R. and J.E. Sadler, 1993. Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. *Blood* 81:(3) 683-689.

Lentz, S.R., and J.E. Sadler. 1991. Inhibition of thrombomodulin surface expression and protein C by the thrombogenic agent homocysteine. *J. Clin. Invest.* 88:1906-1914.

Lewis, C.A., N. Pancharuniti and H.E. Sauberlich, 1992. Plasma folate adequacy as determined

by homocysteine level. *Ann. NY. Acad. Sci.* 669:360-362.
Mandel, H., B. Brenner, M. Berant, N. Rosenberg, N. Lanir, C. Jakobs, B. Fowler, and U. Seligsohn. 1996. Coexistence of hereditary homocystinuria and factor v leiden - effect on thrombosis. *N. Engl. J. Med.* 334:763-768.
McCully, K.S., 1969. Vascular pathology of homocystinemia: implications for the pathogenesis of arteriosclerosis. *Amer. J. Path.* 56:(1) 111-128.
McCully, K.S., and R.B. Wilson, 1975. Homocystinuria theory of arteriosclerosis: development and current status. *Atherosclerosis Rev.* 11:157-246.
McGill J.J., g. Mettler, D.S. Rosenblatt, and C.R. Scriver. 1990. Detection of heterozygotes for recessive alleles. Homocyst(e)inemia: paradigm of pitfalls in phenotypes. *Am. J. Med. Genet.* 36:45-52.
Minno, G.D, G. Davi, M. Margaglione, F.Cirillo, E. Grandone, G. Ciabattoni, I. Catalano, P. Strisciuglio, G. Andria, C. Patrono, and M. Mancini. 1993. Abnormally high thromboxane biosynthesis in homozygous homocystinuria. *J. Clin. Invest.* 92:1400-1406.
Mohammed R., and M. Lamand. 1986. Cardiovascular lesions in cobal-vitamin B_{12} deficient sheep. *Ann. Rech. Vet.* 17:447.
Mölgaard, J., M.R. Malinow, C. Lassvik, A.-C. Holm, B. Upson, and A.G. Olsson. 1992. Hyperhomocyst(e)inaemia: an independent risk factor for intermittent claudication. *J. Inter. Med.* 231:273-279.
Murdoch, J.C., J.C. Rodger, S.S. Rao, C.D. Fletcher, and M.G. Dunnigan. 1977. Down's syndrome: an atheroma-free model? *Br. Med. J.* 2:226-228.
Mudd, S.H., F. Skovby, H.L. Levy, K.D. Pettigrew, B. Wilcken, R.E. Pyeritz, G. Andria, G.H.J. Boers, I.L. Brombert, R. Cerone, b. Fowler, H. Grobe, H. Schmidt, and L. Schweitzer. 1985. *Am. J. Hum. Genet.* 37:1.
Mudd, S.H., J.D. Finkelstein, F. Irreverre, and L. Laster. 1964. Homocystinuria: An enzymatic defect. *Science.* 143:1443-1445.
Mudd, S.H., 1985. Vascular disease and homocysteine metabolism. *N. Engl. J. Med.* 313:751-753.
Mudd, S.H. and H.L. Levy. 1995. Plasma homocyst(e)ine or homocysteine? *N. Engl. J. Med.* 333:325.
Mudd, S.H., H.L. Levy, and F. Skovby, 1995. Disorders of Transsulfuration. *In* The Metabolic basis of Inherited Disease: C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, editors. New York, NY, McGraw Hill Inc. 1887-1912.
Nishinaga, M., T. Ozawa, and K. Shimada. 1993. Homocysteine, a thrombogenic agent, suppresses anticoagulant heparan sulfate expression in cultured porcine aortic endothelial cells. *J. Clin. Invest.* 92:1381-1386.
Reed, T., R. Malinow, J.C. Christian, and B. Upson. 1991. Estimates of heritability of plasma Homocyst(e)ine levels I aging adult male twins. *Clin. Genet.* 39:425-428.
Refsum, H. S. Helland, and P.M. Ueland. 1989. Fasting plasma homocysteine as a sensitive parameter of antifolate effect: A study of psoriasis patients receiving low-dose methotrexate treatment. *Pharmacol. Ther.* 46:510-520.
Rubba P., F. Faccenda, and P Pauciullo. 1990. Early signs of vascular disease I homocystinuria-a noninvasive study by ultrasound methods in eight families with cystathionine-bete-synthase

deficiency. *Metabolism.* 39:1191-1195.

Selhub, J., P.F. Jacques, P.W.F. Wilson, D. Rush and I.H. Rosenberg, 1993. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA* 270:(22) 2693-2698.

Selhub, J., P.F. Jacques, A.G. Bostom, R.B. D'Agostino, P.W.F. Wilson, A.J. Belanger, D.H. O'Leary, P.A. Wolf, E.J. Schaffer and I.H. Rosenberg, 1995. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *N. Engl. J. Med.* 332: 286-291.

Selhub, J. And J.W. Miller. 1992. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by *S*-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am. J. Clin. Nutr.* 55:131-138.

Stampfer, M.J., R. Malinow, W.C. Willett, L.M. Newcomer, B. Upson, D. Ullmann, P.V. Tishler and C.H. Hennekens, 1992. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *JAMA* 268:(7) 877-881.

Stampfer, M.J. and M.R. Malinow. 1995. Can lowering homocysteine levels reduce cardiovascular risk? *N. Engl. J. Med.* 332:328-329.

Ubbink, J.B. W.J.H. Vermaak, A. Vander Merwe and P.J. Becker, 1992. The effect of blood -sample aging and food consumption on plasma total homocysteine levels. *Clinica Chimica Acta* 207:119-128.

Ubbink, J.B., W.J.H. Vermaak, A. Vander Merwe, P.J. Becker, R. Delport, and H.C. Potgieter. 1994. Vitamin requirements for the treatment of hyperhomocysteinemia in humans [1,2]. *J. Nutr.* 124:1927-1933.

Ubbink, J.B., W.J.H. Vermaak, R. Delport, A. Vander Merwe, P.J. Becker, and H. Potgieter. 1995. *Am. J. Clin. Nutr.* 62:802-808.

Ueland, P.M., H. Refsum and L. Brattström. 1992. Plasma homocysteine and cardiovascular disease. *In* Atherosclerotic cardiovascular disease, hemostasis, and endothelial function: R.B. Francis, Jr., editor. New York, Basel, Hong Kong, Marcel Dekker, Inc. 183-236.

Ueland, P.M., H. Refsum, S.P. Stabler, M.R. Malinow, A. Andersson, and R.H. Allen. 1993. Total homocysteine in plasma or serum: methods and clinical applications. *Clin. Chem.* 39:(9) 1764-1779.

Uhlemann, E.R., J.H. TenPas, A.W. Lucky, J.D. Schulman, S.H. Mudd, and N.R. Shulman. 1976. Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. *N. Engl. J. Med.* 295:1283-1286.

Valentine, R.J., H.S. Kaplan, R. Green, D.W. Jacobsen, S.I. Myers, and G.P. Clagett. 1996. Lipoprotein (a), homocysteine, and hypercoagulable states in young men with premature peripheral atherosclerosis: A prospective, controlled analysis. *J. Vasc. Surg.* 23:53-63.

Wall, R.T., J.M. Harlan, and L.A. Harker. 1980. Homocysteine induced endothelial cell injury *in vitro*: A model for the study of vascular injury. *Thromb. Res.* 18:113-121.

Watanabe, M. J. Osada, Y. Aratani, K. Kluckman, R. Reddick, M.R. Malinow, and N. Maeda. 1995. Mice deficient in cystathionine β-synthase: Animal models for mild and severe homocys(e)inemia. *Proc. Natl. Acad. Sci. USA.* 92:1585-1589.

Wilcken, D.E.L., P.B. Nicholas, P.A. Tyrrell, and M.R. Robertson. 1988. Folic acid lowers elevated plasma homocysteine in chronic renal insufficiency: Possible implications for

prevention of vascular disease.*Metabolism*. 7:669-701.
Wilcken, D.E.L. and B. Wilcken. 1976. The pathogenesis of coronary artery disease. *J. Clin. Invest*. 57:1079-1082.
Williams, R.R., M.R. Malinow, S.C. Hunt, B. Upson, L.L. Wu, P.N. Hopkins, B.N. Stults, and H. Kuida. 1990. Hyperhomocyst(e)inemia in Utah siblings with early coronary disease. *Coronary Artery Dis*. 1:681-685.
Wouters, G.A. J., G.H.J. Godfried, H.J. Blom, F.J.M. Trijbels, C.M.G. Thomas, G.F. Brom, R.P.M. Steegers-Theunissen, and T.K.A.B. Eskes. 1993. Hyperhomocysteinemia: as risk factor In women with unexplained recurrent early pregnancy loss. *Fertility and Sterility*. 60:820-825.