Low Circulating Folate and Vitamin B_6 Concentrations

Risk Factors for Stroke, Peripheral Vascular Disease, and Coronary Artery Disease

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Background—A high plasma homocysteine concentration is a risk factor for atherosclerosis, and circulating concentrations of homocysteine are related to levels of folate and vitamin B_6. This study was performed to explore the interrelationships between homocysteine, B vitamins, and vascular diseases and to evaluate the role of these vitamins as risk factors for atherosclerosis.

Methods—In a multicenter case-control study in Europe, 750 patients with documented vascular disease and 800 control subjects frequency-matched for age and sex were compared. Plasma levels of total homocysteine (before and after methionine loading) were determined, as were those of red cell folate, vitamin B_{12}, and vitamin B_6.

Results—In a conditional logistic regression model, homocysteine concentrations greater than the 80th percentile for control subjects either fasting (12.1 μmol/L) or after a methionine load (38.0 μmol/L) were associated with an elevated risk of vascular disease independent of all traditional risk factors. In addition, concentrations of red cell folate below the lowest 10th percentile (<513 nmol/L) and concentrations of vitamin B_6 below the lowest 20th percentile (<23.3 nmol/L) for control subjects were also associated with increased risk. This risk was independent of conventional risk factors and for folate was explained in part by increased homocysteine levels. In contrast, the relationship between vitamin B_6 and atherosclerosis was independent of homocysteine levels both before and after methionine loading.

Conclusions—Lower levels of folate and vitamin B_6 confer an increased risk of atherosclerosis. Clinical trials are now required to evaluate the effect of treatment with these vitamins in the primary and secondary prevention of vascular diseases. **(*Circulation*. 1998;97:437-443.)**

Key Words: atherosclerosis ■ cerebrovascular disorders ■ coronary disease ■ peripheral vascular disease ■ risk factors

An increased plasma homocysteine concentration is associated with premature arterial disease[1–16] and may reflect deficiency states of folate, vitamin B_{12}, or vitamin B_6[17–19] or of certain essential enzymes.[20–25] The relationship between these B vitamins and vascular diseases, however, remains poorly defined. The present study demonstrates that lower circulating levels of folate and vitamin B_6 are often seen in patients with atherosclerosis and confer an increased and independent risk of cardiovascular disease.

Methods

Case Subjects

Patients with clinical evidence of coronary artery disease, peripheral vascular disease, or cerebrovascular disease confirmed by standard diagnostic techniques were included. The inclusion and exclusion criteria have been reported extensively elsewhere.[15] Briefly, 750 case subjects with vascular disease and 800 control subjects younger than 60 years of age, of both sexes, were recruited at 19 centers in nine European countries. Case subjects had defined clinical and objective investigational evidence of vascular disease. Newly or recently diagnosed case subjects were recruited wherever possible, and 69% were recruited within 1 year of diagnosis. Exclusion criteria for both case and control subjects included nonatherosclerotic vascular disease, cardiomyopathy, diabetes mellitus, pregnancy, recent (within 3 months) systemic illness, and psychiatric illness. Conditions thought to influence homocysteine concentrations, such as renal or thyroid

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Received June 5, 1997; revision received November 17, 1997; accepted November 17, 1997.

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TABLE 1. Clinical Data in 750 Case Subjects With Vascular Disease and 800 Control Subjects

Variable	Case Subjects	Control Subjects	P
Male sex, %	73	71	.574
Mean age (SE), y	47.2 (0.31)	43.9 (0.36)	<.001
Mean weight (SE), kg	74.7 (0.46)	73.9 (0.45)	.046
Current smoker, %	54	33	<.001
Hypertension, %	38.5	12	<.001
Hypercholesterolemia, %	53	36	<.001
Mean creatinine (SE), μmol/L	70.8 (0.61)	69.3 (0.44)	.057

disease, anticonvulsant therapy, and recent (<3 months) exposure to nitrous oxide, also served as exclusion criteria.

Control Subjects

Control subjects were clinically healthy and free of overt disease. Where possible, subjects were recruited from a geographic background similar to that of case subjects. Community-based control subjects from random population samples, family practice registers, and occupational registers were considered ideal sources. Just less than half of these subjects came from community samples, one third were recruited from employee health insurance registers, and one sixth were hospital employees. Two percent of control subjects were hospital patients. Control subjects recruited from the three main sources were similar in terms of the major variables studied and plasma total homocysteine (tHcy) levels.

Risk Factors for Vascular Disease

Age, sex, smoking habits, blood pressure, lipid concentrations, weight, and both drug and vitamin usage were documented in all subjects and are shown in Table 1.

Methionine-Loading Test

A methionine-loading test was performed on all subjects in standard fashion.[26] Blood was drawn into tubes containing EDTA for measurement of fasting tHcy. An oral dose of 0.1 g/kg L-methionine was administered, and blood was drawn again 6 hours later for the postload measurement. We refer to the difference between these two concentrations as the increase in tHcy.

Laboratory Measurements

Homocysteine Assay

Total plasma homocysteine was measured by use of a previously described method involving reduction with sodium borohydride, derivatization with monobromobimane, high-performance liquid chromatography (HPLC) separation, and fluorescence detection.[27] Blinded analyses were performed on all samples that were reanalyzed twice on two separate days. A maximum of 10% difference between the two results, ie, 5% difference from the mean, was allowed. If this was exceeded, the analyses were repeated for a third time. The average of these analyses is presented.

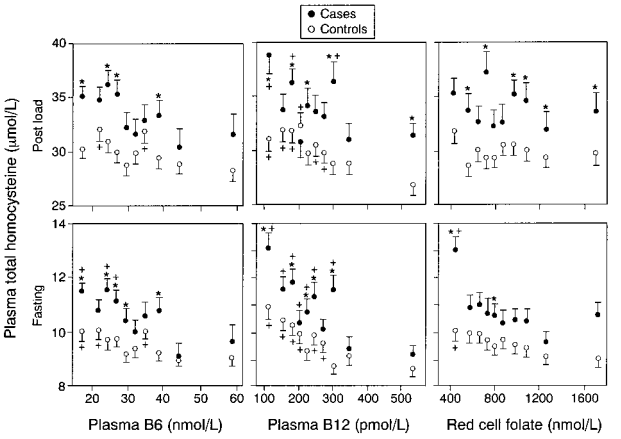
Vitamin Concentrations and Other Assays

Measurements of red cell folate, vitamin B_{12}, vitamin B_6 (measured as pyridoxal 5′-phosphate), and creatinine were performed centrally at Mimelab-AB, Soraker, Sweden. Vitamin B_{12} and folate concentrations were measured by use of a radioimmunoassay technique,[28] and pyridoxal 5′-phosphate was measured by enzymatic photometry with HPLC separation.[29]

Definitions

Traditional Risk Factors

Smokers were defined as those currently smoking any tobacco (at the time of diagnosis for case subjects and at the time of the methionine-loading test for control subjects). Hypertension was considered present if at the time of the methionine-loading test a systolic blood pressure ≥160 mm Hg or a diastolic pressure of 95 mm Hg was observed or if treatment for high blood pressure was administered. For both systolic and diastolic blood pressures, the mean of four values was used (two obtained before and two after the administration of methionine). Hypercholesterolemia was considered present if subjects were taking lipid-lowering drug treatment or had a serum cholesterol ≥6.5 mmol/L (251.4 mg/dL).



Geometric mean and 95% CI bars of fasting and postload homocysteine concentrations in case and control subjects defined by decile cutpoints of folic acid, vitamin B_{12}, and vitamin B_6. Decile cutpoints are based on control samples only. Vitamin values on the *x* axis are the group mean of case and control subjects combined. Case subjects with deficiencies in vitamins other than the one being graphed have been eliminated from the analysis. *Homocysteine level differs significantly ($P<.05$) between case and control subjects in the relevant group. +Case or control homocysteine level is significantly different ($P<.05$) from the level observed in the group above the highest vitamin decile.

Homocysteine Concentrations

For categorical analyses, high tHcy concentrations were defined as levels greater than the 80th percentile for control subjects in both the fasting (12 μmol/L) and the post–methionine-loading state (38 μmol/L). The 80th percentile for control subjects was also used to define an abnormally high increase after methionine loading (27 μmol/L).

Vitamin Deficiencies and Low Vitamin Status

Folate deficiency was defined as a red cell folate concentration <372 nmol/L, which is similar to widely used reference ranges.[30] Low folate status was defined as a concentration below the 10th percentile for control subjects (513 nmol/L). Concentrations of fasting tHcy below this level of folate were higher than those in the upper decile of folate concentration (see Figure). Because this difference persisted when adjusted for deficiencies of both vitamin B_{12} and vitamin B_6, we inferred a functional folate deficiency at and below this level. Vitamin B_{12} deficiency was defined as a plasma concentration <125 pmol/L.[30] Low vitamin B_{12} status was defined arbitrarily as a value below the 10th percentile for control subjects (139.5 pmol/L).

Definitions of vitamin B_6 deficiency are not uniform,[30,31] and values <30[30] or <20 nmol/L[31] may indicate deficiency. In the present study, frank deficiency was defined as <20 nmol/L. Because this was almost identical to the 10th percentile for control subjects (20.8 nmol/L), low vitamin B_6 status was defined as less than the 20th percentile for control subjects (23.3 nmol/L).

Diagnostic Criteria for Vascular Disease

The following criteria were used for the diagnosis of vascular diseases:

TABLE 2. Total Homocysteine Concentrations and Vitamin Levels According to Gender and Case Status

	Men		Women	
Variable	**n**	**Geometric Mean (95% CI)**	**n**	**Geometric Mean (95% CI)**
Fasting tHcy, μmol/L				
Case	544	11.7 (11.3–12.2)	206	10.2 (9.6–10.8)
Control	570	10.2 (10.0–10.4)	230	8.6 (8.3–9.0)
P		<.001		<.001
Postload tHcy, μmol/L				
Case	544	35.0 (34.3–35.7)	206	37.0 (34.9–39.2)
Control	570	31.3 (30.7–31.9)	230	28.1 (27.0–29.2)
P		<.001		<.001
Increase in tHcy, μmol/L				
Case	544	22.8 (21.9–23.7)	206	26.1 (24.7–27.7)
Control	570	20.7 (20.3–21.1)	230	19.1 (18.4–19.9)
P		<.001		<.001
Red cell folate, nmol/L				
Case	514	819 (788–851)	171	727 (686–771)
Control	549	876 (843–911)	226	726 (685–770)
P		.005		.974
Vitamin B_{12}, pmol/L				
Case	543	232 (223–241)	206	248 (234–263)
Control	570	233 (225–243)	230	239 (225–253)
P		.806		.410
Vitamin B_6, nmol/L				
Case	543	26.4 (25.9–26.9)	205	25.7 (24.2–27.2)
Control	570	30.7 (30.2–31.4)	230	32.1 (30.9–33.4)
P		<.001		<.001

tHcy indicates total homocysteine.

1. Coronary heart disease: clinical evidence of angina or myocardial infarction plus a ≥2-fold rise in cardiac enzymes with evolutionary ST-T changes or pathological Q waves alone or angiographic evidence of ≥70% stenosis of a major coronary artery.

2. Cerebrovascular disease: clinical evidence of stroke or transient ischemic attack plus carotid stenosis ≥50% on Doppler or angiography or unequivocal atherosclerotic plaque on angiography or computed tomographic evidence of cerebral infarction without demonstrable source of embolism.

3. Peripheral vascular disease: clinical evidence of intermittent claudication or clearly diminished foot pulses plus obstruction of one major peripheral artery on angiography or Doppler ankle-arm index <0.9.

Statistical Methods

Sample size considerations for this study have been presented elsewhere.[15] Data are presented as mean±SE or percents. When necessary, log transformation was used for skewed variables, and these data are presented as geometric means and 95% CIs. We compared risk factors between case and control subjects using a *t* test or χ^2 test as appropriate. We examined the relationship among tHcy and vitamin concentrations using Pearson correlations. Conditional logistic regression stratified by center, age, and gender was used to investigate models of the risk of coronary artery disease; odds ratios with 95% CIs are reported for these analyses. Differences in tHcy among vitamin deciles were evaluated with ANOVA. A two-sided 5% level of significance is considered significant for all statistical tests; exact probability values are reported down to *P*<.001.

Results

Concentrations of tHcy

Geometric means for fasting, postload, and increase in tHcy values and for the vitamins are shown in Table 2 according to gender and case status. Overall, fasting tHcy values were higher in case subjects than in control subjects in both men and women. Age and weight adjustment had little effect on the values shown in the tables or on significance levels (data not shown). After the methionine-loading test, tHcy values were higher in case subjects than in control subjects, both in men and women. These concentrations also remained high when adjusted for age and weight (data not shown). The increase in tHcy after methionine loading was significantly greater in case subjects than in control subjects in both sexes but was more marked in women than in men. These concentrations also remained high when adjusted for age and weight (data not shown).

Vitamins

Folate concentrations were higher in men than in women. Within men as a group, however, folate levels were lower in case subjects than in control subjects (819.0±1.0 versus 876.2±1.0 nmol/L; *P*=.005; see Table 2). Mean vitamin B_{12}

TABLE 3. Correlations Between Plasma Total Homocysteine and Vitamin Levels in Case and Control Subjects

		Case Subjects		Control Subjects	
Correlation		**Men**	**Women**	**Men**	**Women**
Folate	Fasting tHcy	−.320†	−.251†	−.161†	−.255†
vs	Postload tHcy	−.162†	−.013	−.033	−.146*
	Increase	−.030	.078	.029	−.091
B_{12}	Fasting tHcy	−.273†	−.292†	−.279†	−.233†
vs	Postload tHcy	−.180†	−.149*	−.175†	−.179*
	Increase	−.090*	−.088	−.109*	−.139*
B_6	Fasting tHcy	−.161†	−.252†	−.127*	−.060
vs	Postload tHcy	−.111*	−.254†	−.098*	−.108
	Increase	−.064	−.222†	−.08	−.112
Folate vs vitamin B_{12}		.087*	.160*	.088*	.097
Folate vs vitamin B_6		.005	.099	.137†	.060
Vitamin B_{12} vs vitamin B_6		.086*	.164*	.059	−.030

tHcy indicates total homocysteine.
All data are log transformed.
*$P<.05$; †$P<.001$.

concentrations were similar in both case and control subjects. Vitamin B_6 concentrations were lower in case subjects than in control subjects.

Correlations Between Vitamins and tHcy

The correlations between tHcy and the three vitamins are shown in Table 3 and the Figure.

Fasting tHcy correlated negatively with folate. Postload values correlated negatively with folate in male case subjects and in female control subjects. In contrast, values for the increase in tHcy did not correlate with folate. Fasting, postload, and increases in tHcy values correlated negatively with vitamin B_{12} (see Table 3) in both case and control subjects. The majority of these correlations were significant. Fasting, postload, and increases in tHcy values correlated negatively with vitamin B_6 (see Table 3), and the majority of these correlations were significant. Across the range of vitamin B_6 concentrations, postload tHcy levels were greater in case than control subjects (see Figure).

Vitamin Deficiencies

Prevalences of vitamin deficiencies (defined by use of conventional definitions) and values for the lower 10th and 20th percentiles are shown in Table 4. When a definition of folate deficiency of 372 nmol/L was used, folate deficiency was seen in 2% of control subjects and 4% of case subjects ($P=.048$). Low folate status, corresponding to the 10th percentile for control subjects (<513 nmol/L), was seen in 15% of case subjects ($P=.002$). Prevalences of deficiency of vitamin B_{12} (<125 pmol/L) and low vitamin B_{12} status were no different in case subjects than in control subjects. Deficiency (<20 nmol/L) was seen in 21% of case subjects ($P<.001$). This concentration was almost identical to the 10th percentile for control subjects (20.75 nmol/L; see Table 4). Low vitamin B_6 status (less than the 20th percentile for control subjects, or 23.2 nmol/L) was seen in 35% of case subjects ($P<.001$).

Relationships Between Homocysteine, Vitamins, and Vascular Disease

Variables included in the conditional logistic regression models for vascular diseases included hypertension, smoking, hypercholesterolemia, creatinine, and the concentrations of fasting tHcy, postload tHcy, increase in tHcy, folate, vitamin B_{12}, and vitamin B_6. The results for these analyses are shown in Table 5.

TABLE 4. Prevalence of Low Vitamin Status and Conventionally Defined Vitamin Deficiencies in Case and Control Subjects

	Low Vitamin Level, %		
Variable Threshold	**Case Subjects***	**Control Subjects***	***P***
Vitamin B_6			
10th percentile (20.8 nmol/L)	23	10	<.001
20th percentile (23.3 nmol/L)	35	20	<.001
Deficiency (20.0 nmol/L)	21	8	<.001
Vitamin B_{12}			
10th percentile (140 pmol/L)	12	10	.130
20th percentile (170 pmol/L)	28	20	.328
Deficiency (125 pmol/L)	8	6	.179
Red cell folate			
10th percentile (513 nmol/L)	15	10	.002
20th percentile (604 nmol/L)	26	20	.007
Deficiency (372 nmol/L)	4	2	.048

Cutpoints defining groups are given in parentheses (see "Methods") and are based on control data. Patients with deficiencies in vitamins other than the one being analyzed are eliminated from the analysis.

*For vitamin B_6, n=748 case subjects and 800 control subjects; for vitamin B_{12}, n=749 case subjects and 800 control subjects; and for red cell folate, n=685 case subjects and 775 control subjects.

TABLE 5. Adjusted Odds Ratio of Vascular Disease in Subjects With High Total Homocysteine or Low Vitamin Levels Relative to Subjects With Normal tHcy or Vitamin Levels

Variable	Additional Adjustment	RR (95% CI)	P
High fasting tHcy	· · ·	1.96 (1.49–2.58)	<.001
	Vitamin levels	1.69 (1.26–2.26)	.001
High postload tHcy	· · ·	1.82 (1.39–2.40)	<.001
	Vitamin levels	1.62 (1.2–2.16)	.001
High increase in tHcy	· · ·	1.41 (1.06–1.86)	.017
	Vitamin levels	1.28 (0.96–1.72)	.094
Folate <10th percentile*	· · ·	1.50 (1.03–2.20)	.045
	Fasting tHcy	1.38 (0.93–2.03)	.108
	Postload tHcy	1.45 (0.99–2.13)	.060
	Increase in tHcy	1.50 (1.02–2.20)	.038
B_{12} <10th percentile*	· · ·	1.19 (0.80–1.76)	.392
	Fasting tHcy	1.09 (0.73–.63)	.670
	Postload tHcy	1.16 (0.78–1.72)	.481
	Increase in tHcy	1.17 (0.79–1.73)	.440
B_6 <20th percentile*†	· · ·	1.84 (1.39–2.42)	<.001
	Fasting tHcy	1.76 (1.33–2.34)	<.001
	Postload tHcy	1.79 (1.35–2.37)	<.001
	Increase in tHcy	1.81 (1.37–2.40)	<.001

tHcy indicates total homocysteine; RR, relative risk.

Analyses are stratified by center, age, and gender. All models include hypertension, smoking status, hypercholesterolemia, and creatinine. All three vitamin levels are simultaneously included in models to adjust for their combined influence.

*Also adjusted for lower levels of the other two vitamins.

†The 20th percentile for vitamin B_6 levels is given here because the conventional definition of deficiency (<20 nmol/L) and the 10th percentile (20.75 nmol/L) were virtually identical. Odds ratio of vascular disease was in fact elevated at all three levels (deficient and less than the 10th and 20th percentiles).

Homocysteine

Odds ratios for vascular disease for tHcy have already been reported, adjusted for conventional risk factors.[16] High fasting, increase, and postload tHcy concentrations were significant risk factors for vascular disease after adjustment was made for traditional risk factors and vitamins (see Table 5).

Vitamins

When a conventional definition (<372 nmol/L) was used, folate deficiency was not associated with an increased odds ratio of vascular disease (1.12; CI, 0.52 to 2.41; $P=.77$; data not shown in Table 5). A level of folate below the lowest decile (513.0 nmol/L) conferred an odds ratio of 1.50 (CI, 1.03 to 2.20; $P=.045$; see Table 5) for vascular disease, adjusted for traditional risk factors. When adjusted for fasting tHcy but not the increase or postload values, this was no longer significant.

Neither vitamin B_{12} deficiency (data not shown) nor low vitamin B_{12} status was associated with a significant likelihood of vascular disease (see Table 5). An increased odds ratio of vascular disease was seen both with vitamin B_6 deficiency (not shown in Table 5) and low vitamin B_6 status (odds ratio, 1.84; CI, 1.39 to 2.42; $P<.001$). The risk associated with low vitamin B_6 status persisted when adjusted for the concentrations of tHcy (fasting, postload, or increase; see Table 5).

Discussion

Increases in plasma concentrations of homocysteine are common in patients with stroke, coronary disease, and peripheral vascular disease and confer an independent risk of atherosclerosis.[1–16] In the present study, important links between homocysteine, low vitamin concentrations, and vascular disease risk were seen. The causes of hyperhomocysteinemia in these patients are poorly understood, although reduced activity of cystathionine β-synthase[2,4] or methylenetetrahydrofolate reductase,[24,25] which are essential for the metabolism of homocysteine, could play a role. More importantly, however, concentrations of homocysteine rise as the levels of folate, vitamin B_{12}, and vitamin B_6 fall,[21,22] and high homocysteine concentrations are often seen with deficiency of these vitamins.[17–19]

In this investigation, homocysteine levels were higher in men, although the postload increase was greater in case subjects, with a consequently greater value in total homocysteine level. The gender difference may be because of the fact that more homocysteine is formed in men than in women in conjunction with creatine-creatinine synthesis.[32] It is also possible that there are gender differences in the transsulfuration and remethylation of homocysteine, with more efficient remethylation in women and more efficient transsulfuration in men. Men may therefore have a higher folate requirement. Indeed, in the present study, folate levels were lower in women than in men, and case-control differences were only apparent in men.

In the present study, homocysteine correlated negatively with all three vitamins, although the rise in homocysteine was steepest with lower vitamin levels. When a standard definition (372 nmol/L) was used, folate deficiency was not associated with an increased risk of vascular disease. Low folate status, however, was associated with an increased risk of vascular disease. This risk was reduced by the inclusion of fasting homocysteine in the model, implying that the increased risk of vascular disease accompanying lower folate levels may be explained by the higher circulating homocysteine concentrations. These findings are consistent with those of Pancharuniti et al,[12] who showed an association between lower folate levels and angiographic evidence of ≥50% occlusion of one or more major coronary arteries in white males younger than 50 years of age. Recently, Morrison et al[33] reported a higher 15-year coronary mortality rate in patients with lower folate concentrations. In their study, however, no data were available on homocysteine levels. Our findings are consistent with the observation that low functional levels of folate and other B vitamins, including vitamin B_6, that are prevalent in the general population[19] are also commonplace in patients with atherosclerosis. Because it is possible to lower homocysteine levels with folic acid, such treatment may reduce the risk of atherosclerosis.[16]

Concentrations of vitamin B_6 were lower in case subjects than in control subjects, and deficiency was common (>20%). These findings are unlikely to be a consequence of vascular disease because although vitamin B_6 levels may fall after

myocardial infarction,[34,35] concentrations return to baseline levels after 3 to 4 days.[36] Confounding disorders associated with reduced vitamin B_6 levels, such as cancer, renal disease, diabetes, or alcoholism,[31] also could not have been responsible because such patients had been excluded from the present study.[15] Control subjects had also been selected carefully, and values for random population control subjects were similar to those seen in control subjects recruited from other sources. The large sample size permitted the exploration of a number of models of vitamin B_6 deficiency and low vitamin B_6 status that confirmed the increased relative risk of vascular disease with lower vitamin concentrations. Risk fell with rising vitamin B_6 concentrations and was independent of traditional risk factors. Adjustment for fasting, postload, and increase in homocysteine concentrations did not abolish this effect. High homocysteine concentrations often follow a methionine load[1–4,16,21,22] and have been ascribed to cystathionine β-synthase deficiency.[2,4] In such patients, however, deficiency of vitamin B_6 may be a more satisfactory explanation, because the loading test may be abnormal in such case subjects[31] and the gene frequency for cystathionine β-synthase deficiency is low.[20,36] Other studies have also pointed to an increase in coronary artery disease risk with lower vitamin B_6 concentrations.[14,33] In the study of Selhub et al,[13] a relationship between lower vitamin B_6 levels and carotid disease was seen that diminished when adjusted for homocysteine. In other studies,[37,38] arterial lesions have been seen in animals given pyridoxine-deficient diets. The mechanism for the vascular damage is unclear, although vitamin B_6 may alter platelet function,[39,40] cholesterol concentrations,[41] and antithrombin III activity[41] as well as homocysteine concentrations.[2,3,42–44]

In summary, low concentrations of folate and vitamin B_6 are often associated with high homocysteine concentrations. Lower levels of both these vitamins confer an increased risk of vascular disease. This risk may be mediated through homocysteine in the case of folate but not in the case of vitamin B_6. Such vitamin levels are commonplace in the population and include many individuals now thought to have vitamin concentrations in a normal range. The abnormalities may be readily reversed by folic acid either alone[45,46] or in combination with vitamins B_{12} and B_6.[44,47] Intervention studies are now required to test the effects of such treatment on the primary and secondary prevention of vascular disease.

Appendix

Other Investigators in the European Concerted Action Project

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Acknowledgments

We acknowledge with gratitude the funding for this work partially supplied by the following research organizations: Irish Heart Foundation; The Irish Health Research Board; The Norwegian Council on Cardiovascular Diseases and the Norwegian Research Council; The Netherlands Organization for Scientific Research; The Foundation for Metabolic Research of Utrecht; The Northern Ireland Chest Heart and Stroke Association; Instituto Nacional de Investigacao Sanitarias de la Seguridad Social; FIS No. 92/0914E, Spain; Progetto strategico "Farmaci per malattie orfane" nell'ambito del sottoprogetto Omocistinuria, CNF Roma, Consiglio Nazionale delle Ricerche, Rome, Italy; S.A. Roche, Brussels; The Swedish Heart and Lung Foundation; The Swedish Medical Research Council; and several other bodies.

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