

## Diet, serum homocysteine levels and ischaemic heart disease in a Mediterranean population

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Homocysteine (Hcy) is recognised as a risk factor for IHD. Serum Hcy is negatively correlated with serum folate levels, the main sources of which are fruits, vegetables and legumes. The present case–control study was designed to examine the relationship between serum Hcy levels and IHD and to assess the role of dietary factors in the southern Mediterranean population of Crete, Greece. Serum Hcy, folate, vitamin B<sub>12</sub>, creatinine and glucose levels and a full lipid profile were measured in 152 patients with established IHD, median age 64 (range 33–77) years, and 152 healthy control subjects, age- and sex-matched. Dietary data were assessed using a 3 d food intake record. Compared with controls, patients with IHD had significantly higher daily intakes of vitamin B<sub>12</sub> and MUFA and significantly lower intakes of carbohydrate, fibre, folate, cholesterol, *n*-3 fatty acids and total *trans* unsaturated fatty acids. Moreover, patients had significantly higher serum Hcy, vitamin B<sub>12</sub> and creatinine levels, but significantly lower folate. Serum folate concentrations in both groups had a significant positive correlation with dietary fibre consumption and a significant inverse correlation with vitamin B<sub>12</sub> intake. IHD patients should be encouraged to increase their daily dietary intake of fibre, folate and *n*-3 fatty acids, which are significant components of the traditional Cretan Mediterranean diet. Where dietary folate intake is inadequate, folate supplements are recommended to reduce elevated Hcy levels.

### Homocysteine: Cretan Mediterranean diet: Folate: Ischaemic heart disease

Elevated plasma homocysteine (Hcy) levels are recognised as a conditional risk factor for atherothrombotic vascular disease in coronary, peripheral, and cerebrovascular arterial circulation (Grundey *et al.* 1999; Weiss *et al.* 2002). Hcy is an intermediary amino acid formed by the conversion of dietary methionine to cysteine (Nguyen & McLaughlin, 2002) and is catabolised either by remethylation to methionine or by trans-sulfuration to cystathionine (Haynes, 2002). Remethylation can occur either by methionine synthase (folate and vitamin B<sub>12</sub> dependent), or by betaine-homocystaine methyltransferase; the methyl donor being 5-methyl-tetrahydrofolate (a derivative of folic acid), and betaine, respectively. Trans-sulfuration is catalysed by cystathionine β-synthase and requires the cofactor vitamin B<sub>6</sub> (Cook *et al.* 2002; Haynes, 2002).

The atherogenic mechanism of Hcy-induced vascular damage is still not clearly understood. It is assumed that Hcy is toxic to the vascular wall, causing endothelial dysfunction (Woo *et al.* 1997). Elevated Hcy levels may stimulate the proliferation of vascular smooth muscle cells and impair endothelial function (Welch & Loscalzo,

1998). The interaction of Hcy and endothelial cells may also be followed by platelet activation and thrombus formation (Thambyrajah & Townend, 2000).

Dietary and environmental factors may modify serum Hcy concentrations. Among the factors known to influence Hcy metabolism are several nutrients, including folate, vitamin B<sub>12</sub> and vitamin B<sub>6</sub> (Stein & McBride, 1998). Nutritional deficiencies of these cofactors are associated with hyperhomocysteinaemia (Welch & Loscalzo, 1998; Eikelboom *et al.* 1999). An inverse relation has been shown between serum Hcy levels and the dietary intake of folate and vitamin B<sub>12</sub> (Selhub *et al.* 1993; Rimm *et al.* 1998).

The traditional Cretan Mediterranean diet has been shown to reduce the risk of cardiovascular events (Kafatos *et al.* 1991; Trichopoulou *et al.* 2003) and has been popularised as a healthy diet (Renaud *et al.* 1995). In particular, the consumption of fruits and vegetables appears to have a protective effect against IHD (Brouwer *et al.* 1999a; Joshipura *et al.* 2001). Cross-sectional analyses from the Framingham Heart Study indicate that the frequent consumption of

certain foods (fruits, vegetables, and cereals) correlates with low plasma levels of Hcy, perhaps as a result of the high folate content of these foods (Tucker *et al.* 1996). Dietary intervention studies, using diets high in fresh fruit and vegetables, have also reported a decrease in plasma Hcy concentration (Broekmans *et al.* 2000; Silaste *et al.* 2003).

Principal components of the Cretan diet are olive oil, fruits, vegetables, legumes, fish, and wine (Kafatos *et al.* 1997). The benefits of this diet go beyond its antioxidant properties, since high intakes of plant foods rich in folate, vitamins B<sub>12</sub> and B<sub>6</sub> may consequently reduce serum Hcy levels (Appel *et al.* 2000). Recent data suggest that a 5 µmol/l reduction in Hcy should lower vascular disease risk by one-third (Boushey *et al.* 1995).

The aim of the present study was to investigate the association between dietary intake (particularly dietary folate intake), nutritional status, serum Hcy levels and IHD in the southern Mediterranean population of Crete, Greece.

## Methods

### Subjects

We enrolled 152 patients (136 men and sixteen women) with IHD who were admitted to our hospital over the last 2 years. Patients were considered to have IHD if they had at least one of the following: (1) previous myocardial infarction; (2) history of non-Q infarction or unstable angina; (3) positive stress electrocardiogram and/or echocardiogram.

We excluded patients who had a recent myocardial infarction ( $\leq 6$  weeks) (Egerton *et al.* 1996) or renal dysfunction (serum creatinine  $> 15$  mg/l;  $133$  µmol/l). Patients with clinical evidence of malignant disease and/or receiving vitamin supplements or other medications that could interfere with Hcy levels were also excluded.

Major coronary risk factors for each patient, such as age, sex, hypertension, diabetes mellitus, dyslipidaemia, cigarette smoking, and obesity, were recorded.

The control group consisted of 152 healthy subjects (126 men and twenty-six women) matched for age and sex to patients, with no symptoms of IHD, no other concomitant disease, and who did not take vitamin supplements or medication interfering with Hcy levels.

All subjects were residents of rural areas in one county (Heraklion) of the island of Crete, recruited following public invitation by primary care physicians of seven health centres throughout the county.

### Dietary intake

Dietary intake was evaluated by the 3 d weighed food record. Patients and subjects were given detailed oral and written instructions regarding the completion of a 3 d weighed food record consisting of two mid-week and one weekend day. This method is considered to be one of the most practical dietary assessment methods used (Schroder *et al.* 2001).

Dietary intakes were assessed using a food composition database program developed in the Department of Social

Medicine (Preventive Medicine and Nutrition Clinic) of the University of Crete, which includes analyses contributed by Wageningen Agricultural University, the TNO Nutrition and Food Research Institute, The Netherlands (Transfair Programme), and uses the US Department of Agriculture database version 11.1 adjusted for Greek foods.

Patients and controls provided written informed consent according to the Helsinki declaration.

### Definitions

Diabetics were considered as those who had a fasting serum glucose level  $> 1260$  mg/l ( $7.0$  mmol/l), or who were treated with oral antidiabetic agents or insulin.

As all our patients had established IHD, those with serum levels of LDL-cholesterol (LDL-C)  $> 1300$  mg/l ( $3.4$  mmol/l) and/or triacylglycerols (TG)  $> 1500$  mg/l ( $1.7$  mmol/l) were classified as having dyslipidaemia.

Normal Hcy concentrations range from 6 to 12 µmol/l in the fasting state. Hyperhomocysteinaemia was defined as levels greater than 12 µmol/l (Welch & Loscalzo, 1998; Friedman *et al.* 2001).

### Clinical and laboratory investigations

All samples were collected the morning after 10 h of overnight fasting with water only allowed. Serum levels of Hcy, folate and vitamin B<sub>12</sub> were measured using an IM<sub>X</sub> Analyzer (Abbott Labs, Abbott Park, IL, USA). Serum concentrations of total cholesterol, HDL-cholesterol (HDL-C), and TG were measured using an automated chemistry analyser (Olympus AU-600; Olympus Diagnostics Systems, Eastleigh, Hants, UK) with reagents from the same manufacturer. LDL-C was calculated according to the Friedewald formula except for samples with serum TG above 4000 mg/l ( $4.5$  mmol/l), for which LDL-C could not be determined with the method used. Serum apo A1, apo B100, and lipoprotein (a) were measured by immune nephelometry (lipoprotein (a) test, apo A1 test and apo B100 test; Beckman Instruments Inc., Galway, Republic of Ireland). Renal function profiles and serum glucose concentration were determined by standard methods in routine use.

The department of Clinical Chemistry of Crete University Hospital participates in several quality-assurance programmes and has full clinical pathology accreditation.

### Statistical analysis and presentation of results

Values are expressed as medians and ranges. All *P* values are two-tailed. Between-group results were assessed by Mann–Whitney tests. Frequency analysis was by the  $\chi^2$  test, with Yate's correction. Correlation was assessed by Spearman's correlation ( $r_s$ ).

## Results

### Patient characteristics

The characteristics of the 152 patients with IHD and the 152 controls enrolled in the present survey are listed in

Table 1. Hypertension and diabetes were present in eighty-nine (58.6%) and fifty (32.9%) patients, respectively. Twenty patients (13.2%) were on statin therapy.

#### Daily intake of dietary components in patients with ischaemic heart disease and controls

Patients had significantly higher daily vitamin B<sub>12</sub> and MUFA intake, and significantly lower carbohydrate, fibre, folate, cholesterol, *n*-3 fatty acids and total *trans* unsaturated fatty acids intake (Table 2). Additionally, IHD patients had significantly lower daily energy intake compared with controls (Table 2).

#### Serum homocysteine, folate, vitamin B<sub>12</sub> and creatinine levels of ischaemic heart disease patients and controls

Serum Hcy, vitamin B<sub>12</sub> and creatinine levels were significantly higher and folate was significantly lower in patients (Table 3). Furthermore, there was no significant difference in serum Hcy levels between patients receiving or not receiving statins (16.0 v. 13.7 μmol/l).

**Table 1.** Patients' and controls' demographics (Medians and ranges)

	Controls ( <i>n</i> 152)		Patients with IHD ( <i>n</i> 152)	
	Median	Range	Median	Range
Age (years)	63	40–77	64	33–7
Men: <i>n</i>	126		136	
%	82.9		89.5	
Smokers: <i>n</i>	43		70	
%	28.3		46.1**	
BMI (kg/m <sup>2</sup> )	27.1	15.6–41.1	27.9	18.1–36.0

\*\* *P*=0.0014.

#### Lipids profile of ischaemic heart disease patients and controls

Patients had significantly lower serum total cholesterol, LDL-C and HDL-C concentrations, while their TG levels were higher (Table 3). In IHD patients, statin administration did not affect lipid levels significantly (data not shown).

Moreover, they had significantly higher lipoprotein (a) and lower apo A1 and apo B100 serum levels (Table 3).

#### Correlation of serum total homocysteine, folate and vitamin B<sub>12</sub> levels with dietary components

Serum folate concentration significantly correlated with daily fibre consumption (*r*<sub>s</sub> 0.137; *P*=0.019) and inversely with 24 h vitamin B<sub>12</sub> intake (*r*<sub>s</sub> -0.142; *P*=0.015).

There was no significant correlation either between serum Hcy, folate and vitamin B<sub>12</sub> levels and the daily consumption of other dietary products, or between serum and dietary folate and vitamin B<sub>12</sub> levels.

#### Linear regression analysis

Among twenty potential risk factors for IHD (Tables 2 and 3) proceeding to a linear regression analysis, only low serum apo A1, and HDL-C levels, and high serum creatinine concentrations, as well as low daily fibre consumption were independently and significantly associated with the presence of IHD.

#### Discussion

The present study showed that patients with IHD had significantly higher serum Hcy levels compared with controls. This is in agreement with the literature where subjects with elevated Hcy concentration are not only at high risk for

**Table 2.** Daily uptake of dietary components in patients and controls† (Medians and ranges)

	Controls ( <i>n</i> 152)		Patients with IHD ( <i>n</i> 152)	
	Median	Range	Median	Range
Protein (g)	69.4	18.0–210.4	66.0	21.1–140.5
Total fat (g)	103.5	49.1–220.9	105.5	33.2–183.3
Carbohydrate (g)	160.2	43.4–444.8	113.9***	21.1–291.7
Fibre (g)	19.1	4.7–54.4	15.1***	1.3–38.7
Energy (kJ)	8176	3527–15 803	7130***	2770–15 803
Vitamin E (mg)	11.5	1.3–19.3	11.2	2.6–23.9
Vitamin C (mg)	75.3	1.0–360.4	72.5	2.2–334.3
Vitamin B <sub>6</sub> (mg)	1.2	0.2–2.6	1.2	0.3–2.4
Vitamin B <sub>12</sub> (μg)	2.2	0.1–18.7	2.6*	0.2–53.7
Folate (μg)	254	68–1105	180***	34–574
Cholesterol (mg)	224	35–965	189*	22–694
SFA (g)	24.3	9.9–93.1	24.0	8.9–56.9
MUFA (g)	55.5	17.9–96.1	60.3**	14.4–100.3
PUFA (g)	10.5	4.01–31.4	10.4	3.4–30.5
Total <i>trans</i> unsaturated fatty acids (g)	1.1	0.02–7.6	0.8**	0.04–3.4
<i>n</i> -6 fatty acids (g)	9.0	2.8–27.6	8.9	2.0–28.3
<i>n</i> -3 fatty acids (g)	0.7	0.34–4.8	0.6**	0.2–1.8

SFA, saturated fatty acids.

Median value was significantly different from that for the control group: \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001.

† For details of subjects and procedures, see Table 1 and p. 1014.

**Table 3.** Serum levels of homocysteine (Hcy), folate, vitamin B<sub>12</sub>, creatinine and lipid values of ischaemic heart disease patients and controls† (Medians and ranges)

	Controls (n 152)		Patients with IHD (n 152)	
	Median	Range	Median	Range
Hcy (μmol/l)	12.5	6.6–44.7	13.9**	6.6–50.0
Folate (ng/ml)	7.9	2.4–15.5	6.9**	2.5–20.0
Vitamin B <sub>12</sub> (pg/ml)‡	326	91–835	374**	82– > 2000
Creatinine (mg/l)§	8	6–14	9***	6–21
Total cholesterol (mg/l)	2300	1520–3240	2070***	1190–3420
Triacylglycerols (mg/l)¶	1240	400–3810	1450*	410–5810
HDL-cholesterol (mg/l)	500	300–930	370***	170–730
LDL-cholesterol (mg/l)	1540	840–2270	1350***	520–2610
Lipoprotein (a) (mg/l)	120	20–1180	240***	31–1180
Apo A1 (mg/l)	1360	890–2130	1030***	750–1450
Apo B100 (mg/l)	1170	610–1780	1040**	450–1600

Median value was significantly different from that for the control group: \* $P < 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P < 0.001$ .

† For details of subjects and procedures, see Table 1 and p. 1014.

‡ Serum levels of vitamin B<sub>12</sub> greater than 2000 pg/ml (our cut-off limit) are represented here as >2000 pg/ml.

§ To convert mg/l to mmol/l, multiply by 8.84.

|| To convert mg/l to mmol/l, multiply by 0.0026.

¶ To convert mg/l to mmol/l, multiply by 0.00113.

IHD (Stampfer *et al.* 1992; Wald *et al.* 1998; Bots *et al.* 1999; Whincup *et al.* 1999), but also have more extensive coronary atherosclerosis (Vrentzos *et al.* 2004).

Patients with IHD had significantly lower serum folate concentrations and an inverse correlation between serum Hcy and folate levels was shown. Hcy concentrations depend on a series of intracellular metabolic reactions in which folate acts as a substrate (Das, 2003) and even sub-clinical deficiency of this vitamin can increase serum Hcy levels (Haynes, 2002). There was no significant correlation between serum folate levels and the daily dietary intake of folate. Dietary sources of folate in our population covered a wide range of food products apart from fruit and vegetables. Thus the lack of a statistically significant correlation between dietary intake and serum folate levels could possibly be explained by variations in the bioavailability of folate in different foods (Brouwer *et al.* 1999a), and/or by the paucity of accurate chemical analyses of micronutrients in determining dietary intake for some Greek recipes. However, the fact that IHD patients had both significantly lower serum folate concentration, and daily dietary folate intake compared with controls, indicates a relationship between intake and serum concentration. In addition, others found a strong correlation between total folate intake and serum folate levels (Jacques *et al.* 1993; Selhub *et al.* 1993).

The higher serum Hcy levels of our IHD patients could be partly explained by the slight increase of serum creatinine levels. The kidneys play a significant role in Hcy clearance and metabolism since they contain appreciable levels of both trans-sulfuration and remethylation enzymes (Friedman *et al.* 2001). Serum creatinine is a marker of glomerular filtration rate as well as of future cardiovascular events (Mann *et al.* 2003). Glomerular filtration rate values estimated from serum creatinine are inversely correlated with plasma Hcy levels (Ducloux *et al.* 2000).

The daily vitamin B<sub>12</sub> intake of IHD patients was higher than that of controls. Man is unable to synthesise vitamin B<sub>12</sub>, which is derived from animal products, particularly meat. Vitamin B<sub>12</sub> may have a beneficial effect on fasting levels of Hcy (Appel *et al.* 2000), but in our patients serum vitamin B<sub>12</sub> levels were not associated with Hcy levels.

The present data suggest that dietary folate intake had a major influence on fasting Hcy levels, whereas vitamin B<sub>12</sub> had no effect. Interestingly, the majority of our population (84.2% of controls and 97.4% of patients) had a daily folate intake lower than European recommendations (>400 μg/d) (de Bree *et al.* 1997; Hassapidou & Fotiadou, 2001; Kafatos & Codrington, 2001).

IHD patients had a significantly lower dietary fibre intake than controls. Increased dietary fibre intake, from fruits, vegetables, legumes, and cereals, has been associated with a lower risk for IHD (Rimm *et al.* 1996; Liu *et al.* 2002). In the present study, daily fibre consumption significantly correlated with serum folate concentration, as both these components share the same food sources.

Patients and case subjects also had a significantly lower *n*-3 fatty acids intake. Epidemiological studies and controlled clinical trials have demonstrated beneficial effects of *n*-3 fatty acids on CHD (AHA Scientific Statement, 2001; Hu & Willet, 2003). Moreover, Bucher *et al.* (2002) in a meta-analysis suggest that *n*-3 fatty acids intake reduces overall mortality, mortality due to myocardial infarction, and sudden death in patients with IHD. An increased intake of *n*-3 fatty acids from fish, fish oil supplements, or plant sources should be considered essential for IHD patients (Carroll & Roth, 2002).

IHD patients had lower serum total cholesterol, LDL-C, HDL-C, apo B100 and apo A1 and higher TG and lipoprotein (a) serum levels when compared with controls. In a previous study (Papakonstantinou *et al.* 2002), we showed that low HDL-C levels are the principal lipid

abnormality in patients with acute myocardial infarction in the southeast part of Greece. This pattern (low HDL-C and elevated TG) seems to be the lipid pattern of IHD patients in this area. In addition, HDL-C, apo A, and lipoprotein (a) are not directly diet dependent (Katan *et al.* 1995). Finally, the incidence of smoking was higher among IHD patients than controls (46.1 v. 28.3%). The harmful effects of smoking on HDL-C and TG levels are well known (Mikhailidis *et al.* 1998).

The prevalence of cardiovascular disease has increased during the last 30 years in Crete (Voukiklaris *et al.* 1996), a trend that appears to be related to dietary and life-style changes that have been taking place (Kafatos *et al.* 2000). Notable among these have been dramatic reductions in physical activity and also the gradual abandonment of the traditional Cretan diet. The Cretans now have higher saturated fat (+25%) and lower monounsaturated fat (−20%) intakes, indicating increases in the consumption of meat and cheese, and simultaneously decreases in the mean daily consumption of bread, fruits, vegetables, potatoes, and fibre (Kafatos *et al.* 1997). Decreases in vegetable and fruit consumption, both good sources of folate, will reduce folate status and increase Hcy concentrations (Tucker *et al.* 1996).

A recent trial of individuals with established IHD showed that a Mediterranean diet rich in fruits and vegetables and  $\alpha$ -linolenic acid substantially reduced the recurrence of IHD events for up to 4 years compared with a regular low-fat diet (relative risk 0.28; CI 0.15, 0.53). However, the Mediterranean diet did not alter, at least qualitatively, the usual relationships between major risk factors and recurrence (de Lorgeril *et al.* 1999).

In IHD patients, serum Hcy levels are a significant predictor of late cardiac events (Stubbs *et al.* 2000) and increased mortality risk, possibly as a consequence of atherosclerotic changes (Knekt *et al.* 2001), independently of other traditional risk factors (Nygard *et al.* 1997). Wald *et al.* (2002) showed that lowering Hcy concentrations by 3  $\mu\text{mol/l}$  (achievable by increasing folic acid intake) would reduce the risk of IHD by 16% (11 to 20%), deep-vein thrombosis by 25% (8 to 38%), and stroke by 24% (15 to 33%). In our patients it is essential to reduce the high Hcy levels (Vrentzos *et al.* 2000). This could be achieved by increasing the consumption of folate, not only from foods rich in folate (de Lorgeril *et al.* 1994; Singh *et al.* 2002), but also with low doses of folate supplements (Brouwer *et al.* 1999b; Jacques *et al.* 1999; Riddell *et al.* 2000; Rampersaud *et al.* 2003). Adequate dietary folate intake may be recommended for individuals with Hcy >10  $\mu\text{mol/l}$  (de Bree *et al.* 1997; Malinow *et al.* 1999). Treatment with folic acid has been shown to reduce serum Hcy concentration and significantly improve endothelial dysfunction in patients with IHD (Title *et al.* 2000; Verhaar *et al.* 2002; Willems *et al.* 2002), but the effect on cardiovascular morbidity and mortality is as yet unknown (Voutilainen *et al.* 2001).

To conclude, the present data suggest that IHD patients should be encouraged to increase their daily dietary intakes of fibre, folate and *n*-3 fatty acids, which are significant components of the traditional Cretan Mediterranean diet.

Where dietary folate intake is inadequate, folate supplements are recommended to reduce elevated Hcy.

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