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Dose-dependent effects of folic acid on plasma homocysteine in a randomized trial conducted among 723 individuals with coronary heart disease

PACIFIC Study Group*

Aims To determine the effects on homocysteine levels of two doses of folic acid compared to placebo, where the high dose is typical of that provided by pharmacological intervention and the low dose approximates that provided by dietary supplementation.

Methods and Results The PACIFIC study was a double-blind, placebo-controlled, factorial randomized trial. Seven hundred and twenty-three individuals with a history of myocardial infarction or unstable angina were recruited from 28 clinical cardiology centres in Australia and New Zealand and randomized to folic acid 2·0 mg daily, folic acid 0·2 mg daily or placebo. The primary outcome, homocysteine, was measured using a fluorescence polarization immunoassay. Compared to placebo, 2·0mg folic acid reduced homocysteine by 1·8 μmol . l^{-1} [95% confidence interval (CI) 1·3–2·3] and 0·2 mg reduced homocysteine by 1·2 μmol . l^{-1} (95% CI 0·8–1·7). The higher dose reduced homocysteine significantly more than the lower dose (P=0·01).

Conclusions Both doses of folic acid reduced homocysteine, but the effects of the 2·0 mg dose were about one third greater than the 0·2 mg dose. Fortification of foods with folic acid should result in population-wide lower levels of homocysteine but high-dose pharmacological supplementation would produce greater reductions for high-risk individuals.
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Key Words: Homocysteine, folic acid, coronary heart disease, randomized trial.

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Introduction

Epidemiological studies have identified continuous positive associations between blood homocysteine levels and the risk of occlusive vascular disease[1–5]. These associations exist across a broad range of usual homocysteine levels and appear to be independent of other risk factors. A recent overview reported that a 1 μmol . l^{-1} prolonged lower level of blood homocysteine was associated with an approximate 10% lower risk of vascular disease[4]. Blood homocysteine levels are inversely related to folate levels[6] and large increases in usual daily oral folic acid intake (0·5 to 5·0 mg daily) have been demonstrated to reduce blood homocysteine levels by an average of about one quarter[7]. The effects on blood homocysteine levels of smaller increments in folic acid intake that might possibly be achieved through fortification of foods (perhaps 0·2 mg daily), rather than therapeutic intervention are not well established[8–10]. Those individuals likely to obtain the greatest benefit from supplementation of folic acid by either means are those at the greatest absolute risk of cardiovascular disease, such as patients with a history of ischaemic heart disease[11].

The present placebo-controlled randomized trial (PACIFIC: Prevention with A Combined Inhibitor and Folic Acid In Coronary heart disease) was conducted among patients with a history of myocardial infarction or unstable angina, to determine the effects on blood homocysteine levels of two doses of folic acid compared to placebo. Using a 3 × 3 factorial design, the effects of two doses of the vasopeptidase inhibitor, omapatrilat, compared to placebo on blood pressure and neurohormone levels were investigated simultaneously: the results of this comparison will be reported separately.

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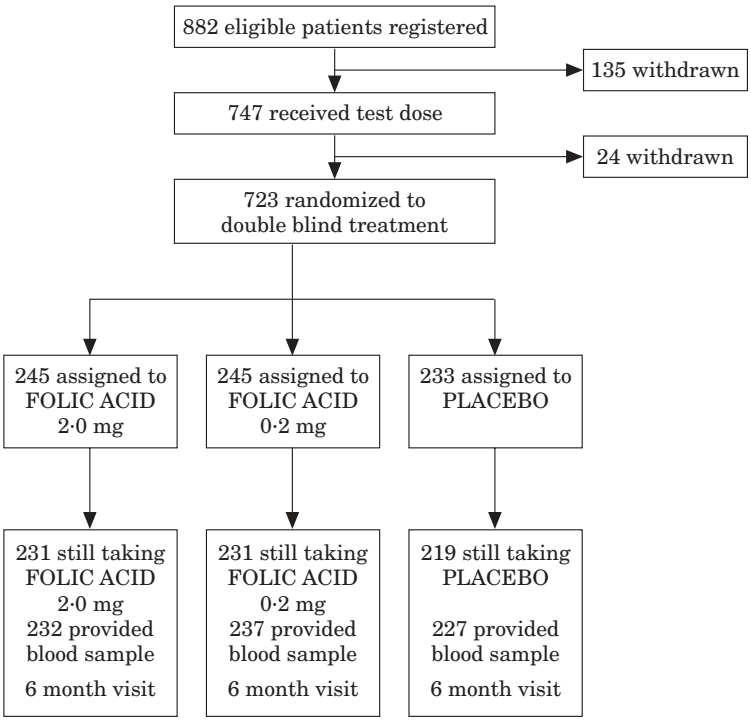

Figure 1 **Trial profile.**

Methods

Study participants

Participants were recruited to the trial from 28 hospitals in Australia and New Zealand. At each hospital, an ethics committee approved the conduct of the study and all participants provided written informed consent. Individuals were potentially eligible for inclusion in the trial if they had: (1) a history of acute myocardial infarction or a hospital admission for unstable angina 2 weeks or more prior to registration; (2) a left ventricular ejection fraction of 40% or more; and (3) a high risk of subsequent myocardial infarction or coronary heart disease death. Individuals were deemed to be at high risk if they were 65 years or older, were current cigarette smokers, were diabetic, had a history of more than one previous myocardial infarction or had a history of Canadian Cardiovascular Society angina grade 2 or above.

Exclusion criteria were based primarily upon eligibility for the omapatrilat component of the study and resulted principally in the exclusion of individuals with a definite indication for, or contraindication to, angiotensin converting enzyme inhibitor therapy. In terms of the folic acid comparison, exclusion of potential participants was on the basis of the responsible investigator's opinion regarding a definite indication for, or contra-indication to, treatment with folic acid.

Study treatment and follow-up

Prior to randomization, all potentially eligible individuals entered a 4-week run-in phase during which they received single-blind treatment with placebo–folic acid and placebo–omapatrilat. The purpose of this phase was to identify individuals who were unlikely to comply with the study follow-up requirements. Those participants who successfully completed the run-in phase were randomized, double-blind in a 3×3 factorial design to one of three folic acid treatment groups (folic acid 2·0 mg, folic acid 0·2 mg or folic acid–placebo) and one of three omapatrilat treatment groups (omapatrilat 40 mg, omapatrilat 20 mg or omapatrilat–placebo). Study treatment assignment was obtained via a telephone call or Internet connection to a central computerized randomization service. Randomization was stratified by clinical centre. Follow-up visits were scheduled for 1 month, 3 months and 6 months after randomization, with a final post-study visit at 7 months, 1 month after the scheduled discontinuation of study treatment.

Blood collection and laboratory assays

The primary study outcome was the blood homocysteine level. The effects of folic acid on blood homocysteine levels were determined from measurements made at randomization and at the 6-month follow-up visit. On each occasion, blood samples were obtained at the same

Table 1 Baseline characteristics of treatment groups

	Folic Acid		Placebo (n=233)
	2·0 mg (n=245)	0·2 mg (n=245)	
Participant characteristics and medical history			
Age, years (SD)	68 (8)	68 (7)	68 (8)
Male (%)	204 (83)	201 (82)	185 (79)
Current smoker (%)	38 (16)	31 (13)	34 (15)
Myocardial infarction (%)	177 (72)	182 (74)	164 (70)
Unstable angina (%)	141 (58)	138 (57)	141 (62)
Diabetes (%)	41 (17)	27 (11)	37 (16)
Vitamin supplement use* (%)	27 (11)	24 (10)	35 (15)
Biochemical and genetic parameters			
Serum folate nmol . l^{-1} (SD)	18·6 (8·2)	19·6 (8·9)	19·4 (8·4)
Red blood cell folate nmol . l^{-1} (SD)	748 (303)	753 (288)	762 (305)
Blood homocysteine μmol . l^{-1} (SD)	11·1 (3·7)	10·9 (3·8)	10·9 (4·0)
Vitamin B_{12}, pmol . l^{-1} (SD)	286 (168)	308 (210)	302 (188)
Serum creatinine, mg . dl^{-1} (SD)	1·0 (0·2)	1·0 (0·2)	1·0 (0·2)
MTHFR C677T genotype (%)			
Cysteine/cysteine	118 (48)	102 (42)	101 (43)
Cysteine/threonine	106 (43)	113 (46)	103 (44)
Threonine/threonine	21 (9)	30 (12)	29 (12)

*Multivitamins or B-complex vitamin supplements.
SD=standard deviation; MTHFR=methylene tetrahydrofolate reductase; C=cysteine; T=threonine.

time of day after the participant had fasted for at least 4 h. Venous blood samples were collected into chilled EDTA vacutainers that were placed immediately on ice and then centrifuged at 4 °C within 20 min. Plasma and buffy coat (at the baseline visit only) were pipetted off and stored at −70 °C for later analysis. Total plasma homocysteine levels were determined from IMX-automated, fluorescence-based enzyme immunoassays[12]. With this method, total free and protein-bound circulating homocysteine moieties are reduced to free homocysteine with dithiothreitol. The free homocysteine is then converted to S-adenosyl-l-homocysteine (SAH) with SAH-hydrolase and excess adenosine and the total homocysteine concentration is determined by fluorescence polarization immunoassay after the addition of an anti-SAH antibody and a fluoresceinated tracer (S-adenosyl cysteine). The results obtained by this method are highly correlated with those obtained by high-performance liquid chromatography (r=0·99)[12]. Deoxy-ribonucleic acid (DNA) for determination of methylenetetrahydrofolate reductase (MTHFR) polymorphisms was extracted from the buffy coat specimens by a salting-out method[13,14]. The extracted DNA was amplified using the polymerase chain reaction and the 677 cysteine (C) to threonine (T) substitution at the MTHFR locus was identified by using the restriction enzyme HinfI[15].

Serum folate, red blood cell folate, serum vitamin B_{12}, serum creatinine, and serum cholesterol levels were determined from venous blood samples analysed at the laboratories of the 28 participating centres. Baseline levels were determined from assays of a non-fasting blood sample collected at registration (4 weeks prior to randomization) and follow-up levels from a fasting sample collected at the 6 month visit.

Statistical analysis

The study sample size of 723 patients per group (233 to 245 patients in each of the three randomised groups) was estimated to provide 80% power with $P=0·05$ to detect a 1·1 μmol . l^{-1} difference between each of the groups in the change in blood homocysteine levels from baseline to 6 months (with the assumption of a standard deviation of change in blood homocysteine levels of 4·5 μmol . l^{-1} within each treatment group). All analyses were conducted according to the intention-to-treat principle. The effects of folic acid on blood homocysteine levels were determined by using analysis of variance to obtain effect estimates and *P*-values for main and interaction effects. The possible effects of covariates on the treatment effects observed in the univariate analyses were investigated using analysis of covariance. Differences in the tolerability of the randomised treatments were tested using the method of Mantel and Haenszel. All *P*-values were calculated from two-tailed test of statistical significance. The principal analyses were conducted using the SAS package (Version 8, SAS Institute Inc, Cary, NC).

Results

A total of 882 potentially eligible participants commenced the run-in phase. Of these, 159 individuals did

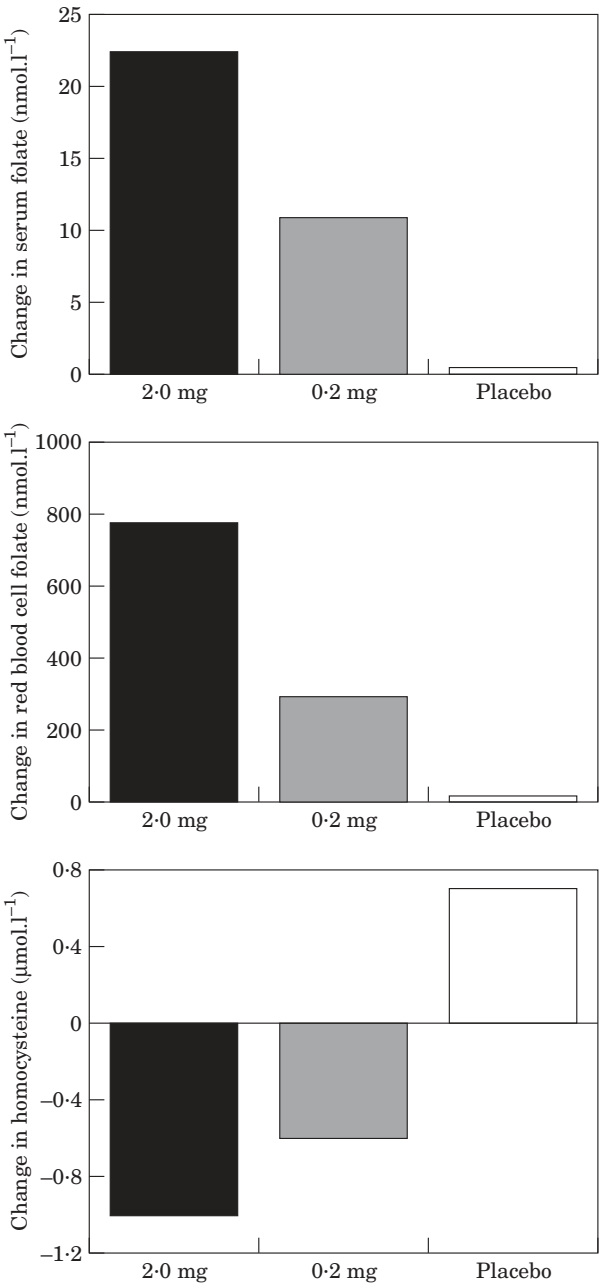

Figure 2 **Effects of folic acid on mean serum folate levels, mean red blood cell folate levels and mean blood homocysteine levels from baseline to 6 months follow-up by randomized group.**

not proceed to randomization (Fig. 1), the principal reasons being failure to meet the study inclusion criteria, likely non-adherence with the study treatment and follow-up regimen and probable intolerance of the factorial omapatrilat arm of the study. Of the 723 randomized participants, 721 survived to the end of the scheduled 6 months of follow-up, 708 (98%) attended the 6-month follow-up visit and 696 (96%) provided a pair of baseline and follow-up blood samples for assay.

The mean age of the 723 randomized participants was 68 years, 79% were aged 65 years or over and 82% were male (Table 1). Overall, the mean blood homocysteine level at baseline was 11·0 μmol . l^{-1}, the mean serum folate level was 19·2 nmol . l^{-1} and the mean red blood cell folate level was 754 nmol . l^{-1}. Of those randomized, 42% had a history of acute myocardial infarction alone, 28% had a history of hospitalization for unstable angina alone and 30% had a history of both. The mean time between the occurrence of the qualifying events and inclusion in the study was 6·7 years for myocardial infarction and 4·4 years for unstable angina. The mean blood pressure at baseline was 133/77 mmHg, the mean body mass index was 27·5 kg . m^2 and the mean total blood cholesterol 186 mg . dl^{-1}. At entry to the study, 12% of randomized participants were using multivitamins or B-complex vitamin supplements (Table 1).

A total of 681 (94%) participants were taking randomized treatment at the 6 months follow-up visit. There was no difference between the rates of withdrawal from treatment in the three randomized groups (all 6%). From baseline to 6 months there was a 22·2 nmol . l^{-1} [95% confidence interval (CI) 20·2–24·3] increase in the serum folate level of the 2·0 mg folic acid group compared to placebo and an 11·2 nmol/l (95% CI 9·2–13·2) increase in the serum folate level of the 0·2 mg folic acid group compared to placebo (Fig. 2). Red blood cell folate levels also rose by 759 nmol . l^{-1} [95% CI 693–825] in those assigned to folic acid 2·0 mg compared to placebo and by 283 nmol . l^{-1} (95% CI 219–347) in those assigned to folic acid 0·2 mg compared to placebo. For both serum folate levels and red blood cell folate levels, the effects of the higher dose of folic acid were significantly greater than the effects of the lower dose of folic acid (both $P<0·001$). Over the same time period there were no significant effects of folic acid on the blood levels of vitamin B_{12}, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol or creatinine (all $P>0·1$).

During the 6-month follow-up period, blood homocysteine levels were reduced in both folic acid treatment groups compared to placebo (Fig. 2). There was a 1·8 μmol . l^{-1} (95% CI 1·3 to 2·3) reduction in blood homocysteine levels in the folic acid 2·0 mg group compared to placebo ($P<0·001$) and a 1·2 μmol . l^{-1} (95% CI 0·8 to 1·7) reduction in blood homocysteine levels in the folic acid 0·2 mg group compared to placebo ($P<0·001$). The reduction in the blood homocysteine level in the folic acid 2·0 mg group compared to that in the folic acid 0·2 mg group was 0·6 μmol . l^{-1} (95% CI 0·1 to 1·1, $P=0·01$).

There was a significantly greater reduction in blood homocysteine levels among individuals with higher baseline homocysteine levels ($P<0·001$) and a borderline significant lesser reduction in blood homocysteine levels among individuals with higher baseline serum folic acid levels ($P=0·05$) (Table 2). Significant differences in the effects of folic acid on blood homocysteine levels were not, however, observed among groups defined at

Table 2 Reduction in plasma homocysteine levels (μmol . l) by baseline subgroups

Participant subgroup*	Number of participants	Difference** μmol . l (95%CI)	*P* for interaction
Gender			
Male	570	1·46 (1·00–1·91)	0·6
Female	126	1·77 (0·83–2·70)	
Age			
Older (>=69 years)	358	1·74 (1·18–2·30)	0·2
Younger (<69 years)	338	1·22 (0·61–1·83)	
Plasma homocysteine			
Higher (>=10 μmol . l^{-1})	382	2·23 (1·69–2·78)	<0·001
Lower (<10 μmol . l^{-1})	314	0·85 (0·29–1·41)	
Serum folate			
Higher (>=17 nmol . l^{-1})	334	1·19 (0·67–1·72)	0·05
Lower (<17 nmol . l^{-1})	362	1·94 (1·40–2·48)	
Red blood cell folate			
Higher (>=690 nmol . l^{-1})	332	1·19 (0·60–1·78)	0·1
Lower (<690 nmol . l^{-1})	364	1·89 (1·29–2·48)	
Vitamin B_{12}			
Higher (>=262 pmol . l^{-1})	347	1·21 (0·63–1·78)	0·2
Lower (<262 pmol . l^{-1})	349	1·81 (1·23–2·39)	
Vitamin supplements***			
Present	82	0·98 (0·18–1·78)	0·3
Absent	614	1·58 (1·13–2·03)	
MTHFR C677T genotype			
Cysteine/Cysteine	304	1·53 (0·91–2·16)	
Cysteine/Threonine	313	1·40 (0·79–2·01)	0·7
Threonine/Threonine	79	1·99 (0·81–3·17)	
Overall	696	1·51 (1·10–1·92)	

*Continuous variables were dichotomized by median values.
**Difference between actively treated group (folic acid 2·0 mg and 0·2 mg combined) and placebo group in change from baseline to 6 months.
***Multivitamins or B-complex vitamin supplements.
CI=confidence interval; MTHFR=methylene tetrahydrofolate reductase; C=cysteine; T=threonine.

baseline by red blood cell folate levels, vitamin B_{12} levels, supplemental vitamin use, age or gender (all $P>0·1$). There was no significant interaction of the methylene tetrahydrofolate reductase (MTHFR) polymorphism with the effects of randomized treatment ($P=0·7$). The T/T MTHFR polymorphism was, however, associated with a higher baseline level of homocysteine than either the T/C or C/C polmorphisms (T/T — 12·4 μmol . l^{-1}; T/C — 10·8 μmol . l^{-1}; C/C — 10·8 μmol . l^{-1}, both comparisons with T/T polymorphism $P<0·003$).

Over the duration of the study, while both doses of folic acid reduced blood homocysteine levels compared to placebo, the blood homocysteine levels in those participants assigned placebo folic acid rose by about 0·7 μmol . l^{-1}. This increase appears to be due to the omapatrilat therapy administered in the factorial arm of the study, with blood homocysteine levels significantly increased by both doses of omapatrilat compared to placebo–omapatrilat (40 mg vs placebo, 0·8 μmol . l^{-1}, $P<0·001$; 20 mg vs placebo, 0·7 μmol . l^{-1}, $P=0·006$).

Discussion

This study demonstrates that folate supplementation at a dose of either 0·2 mg or 2·0 mg daily reduces blood homocysteine levels among high-risk patients with a history of coronary heart disease. The reduction in homocysteine levels achieved with the higher level of supplementation was significantly greater than that achieved with the lower dose. If the ongoing large-scale trials confirm the beneficial effects of folic acid supplementation on cardiovascular disease anticipated from observational studies[4], the most effective strategy for the prevention of homocysteine-related cardiovascular morbidity and mortality is likely to be one that; (1) achieves the maximum possible population-wide fortification of foodstuffs and (2) targets additional supplementation at those individuals at greatest risk of cardiovascular events.

In the PACIFIC study, in addition to a lesser effect of the lower dose of folic acid, the proportional reduction in blood homocysteine levels (15%) achieved with the

higher dose of folic acid was also somewhat smaller than that typically reported by previous trials (about 25%)[7]. Rates of non-adherence to study treatment in the PACIFIC study were low and few participants used non-study vitamin supplements, so 'drop-out' and 'drop-in' are likely to have resulted in only minor underestimation of the effects of folic acid on blood homocysteine levels. The most likely explanation of the lesser proportional reduction in homocysteine levels observed in the PACIFIC study is the low mean baseline blood homocysteine level, and the high mean baseline folate levels among the PACIFIC study participants. Both of these parameters were associated with the magnitude of the homocysteine lowering effect achieved in the PACIFIC study and both were identified as important sources of heterogeneity between the treatment effects observed in previously completed trials[7]. Heterogeneity of treatment effects was not observed by MTHFR polymorphism, despite higher mean blood homocysteine levels among those with the T/T polymorphism, most likely reflecting the low statistical power for the comparisons. The reasons for the low baseline blood homocysteine levels and the high baseline blood folate levels among the PACIFIC study participants are uncertain, but may include healthy changes in lifestyle following the initial diagnosis of coronary heart disease, the use of vitamin B supplements and the blood collection methodology employed (leakage of homocysteine from red blood cells was minimized by rapid processing of the blood samples).

The increase in blood homocysteine levels that was observed in the group assigned folic acid–placebo appears to be due to the omapatrilat treatment studied in the factorial comparison of this trial. The effects of omapatrilat, or any other blood pressure lowering drug, on serum homocysteine levels have not previously been documented and the physiological explanation for the 5–10% higher homocysteine levels among omapatrilat-treated participants remains uncertain. Omapatrilat did not influence the levels of serum folate, red blood cell folate or vitamin B_{12}. Cross reactivity of omapatrilat with the homocysteine assay is unlikely but altered renal metabolism of homocysteine resulting from an omapatrilat-induced decrease in glomerular filtration rate is a possible explanation[16]. Over the 6-month treatment period, plasma creatinine levels rose by 4% in each of the omapatrilat groups compared to placebo ($P<0{\cdot}001$ for both comparisons). If differential effects of blood pressure lowering agents on homocysteine levels are identified in future studies, this might have important implications for both the selection of blood pressure lowering therapy and the use of folic acid.

Folic acid appears to be an inexpensive and effective means of lowering blood homocysteine levels. Using a combination of fortified foods and targeted therapeutic intervention it should be possible to achieve moderate reductions in the mean blood homocysteine level of the population and marked reductions in the mean blood homocysteine level of those at high cardiovascular risk. Prior to the implementation of such strategies, the findings of large-scale randomized trials of the effects of folic acid on major morbidity and mortality are required to fully assess the overall balance of risks and benefits associated with folic acid supplementation.

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Appendix

This manuscript is dedicated to Nicholas Dudman who died during the period of the study. *PACIFIC Management Committee*: Nicholas Dudman (deceased), David Hunt, Stephen MacMahon, Bruce Neal, Mark Richards, John Simes, Andrew Tonkin, David Wilcken; *Project Managers*: Judy Murphy, Yvonne Cleverly; *Data Safety Monitor*: Anthony Keech; *Writing Committee*: Bruce Neal,

Stephen MacMahon, Takayoshi Ohkubo, Andrew Tonkin, David Wilcken; *Coordinating Centres*: Clinical Trials Research Unit, University of Auckland, New Zealand (Kathy Bos, Hazel Bartram, Derrick Bennett, Joanna Broad, Deanne Douglas, Sheila Fisher, Barry Gray, Amanda Milne, Alan McCulloch, Colleen Ng, Megan Pledger, Adrienne Pryor, Aimee Santos, Alex Slater, Karen Yiu) and Institute for International Health, University of Sydney, Australia (Alan Brnabic, Stephen MacMahon, Bruce Neal, Mark Woodward); *Clinical Centres*: Australia — Austin & Repatriation Medical Centre (Louise Burrell, Louise Brown), Box Hill Hospital-(Angas Hamer, Louise Roberts), Canberra Hospital (Ian Jeffery, Pearle Taverner, Alice Kam), Flinders Medical Centre (Philip Aylward, Fiona Wollaston, Anthony Whitehead), Fremantle Hospital and St John of God Hospital Murdoch (Geoffrey Lane, Gill Tulloch, Nicole Forrest, Jan Garrett), Gold Coast Hospital (Greg Aroney, Pam Hicks), John Hunter Hospital (Jonathan Silberberg, Anne Gordon, Julie Holliday, Liz Hicks), Launceston General Hospital (Bhuwan Singh, Carol Singh, Judi Wilken), Northern Hospital (Bruce Jackson, Gloria Rudge), Prince Charles Hospital (Malcolm West, Anne Carle), Princess Alexandra Hospital (Thomas Marwick, Cindy Hall), Royal Brisbane Hospital (David Cross, Daphne Craw), Royal Melbourne Hospital (David Hunt, Michelle Sallaberger), The Queen Elizabeth Hospital (John Horowitz, Matthew Worthley, Liz Owen), Wollongong Hospital (Dwain Owensby, Bill McKenzie; Julie Kesby-Smith, Suzanne MacKinley); New Zealand — Auckland Hospital (Norman Sharpe, Robert Doughty, Jenne Pomfret), Christchurch Hospital (Mark Richards, Rona Buttimore, Lorraine Skelton), Dunedin Hospital (Michael Williams, Ralph Stewart, Marguerite McLelland), Greenlane Hospital (Harvey White, Mary Denton), Hastings Study Centre (Richard Luke, Jane Kenyon), Hutt Hospital (Stewart Mann, Anne Cuthbert), Foxglove Clinic (Stuart Reubin, Pam Yorke), Middlemore Hospital (Douglas Scott, Miles Williams, Renee Coxon), Nelson Hospital (Darryl Fry, Malcolm Clark, Pauline Barclay), North Shore Hospital (Hamish Hart, Carol O'Toole, Philippa Wright); Tauranga Study Centre (Leigh Nairn, Jeannie Bruning); Waikato Hospital (Denis Friedlander, Liz Low); *Financial support:* This study was funded by a grant from Bristol Myers Squibb but was designed and conducted by the investigators independently of the principal sponsor. Stephen MacMahon is the recipient of an Eccles Award from the National Health and Medical Research Council of Australia and a program grant from The Medical Foundation of the University of Sydney. Mark Richards holds the National Heart Foundation (NZ) Chair of Cardiovascular Studies and is Director of a Program Grant from the New Zealand HRC. Takayoshi Ohkubo is a recipient of an overseas research fellowship from the Japanese Heart Foundation. *Endorsement:* This study was conducted under the auspices of the National Heart Foundation of Australia and the National Heart Foundation of New Zealand.