PROTOCOL

*F*olic *A*cid for *V*ascular *O*utcome *R*eduction *I*n *T*ransplantation
(FAVORIT)

A Randomized, Controlled Clinical Trial of the Effect of a High Dose Combination of Folic Acid, Vitamin B6 and Vitamin B12, on Arteriosclerotic Cardiovascular Disease Outcomes in Chronic, Stable Renal Transplant Recipients

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This document is a summary of the key elements of the FAVORIT study. For details, it is essential to consult the Manual of Operations. This document can be obtained from:

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SUMMARY

This multicenter, randomized, double-blind controlled clinical trial has been designed to determine whether total homocysteine (tHcy)-lowering treatment with a standard multivitamin augmented by a high dose combination of folic acid, vitamin B12, and vitamin B6, versus treatment with an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12., reduces the pooled rate of recurrent and de novo cardiovascular disease [CVD] outcomes (i.e., pooled occurrence of non-fatal and fatal arteriosclerotic outcomes, including coronary heart, cerebrovascular, and peripheral vascular disease events = primary outcome), among clinically stable renal transplant recipients (RTRs) who have mild to moderately elevated tHcy levels. The basic eligibility criteria are age 35 to 75 years old, functioning renal allograft for ≥ 6-months with serum creatinine based glomerular filtration rate (GFR) ≥ 30 mL/min for men and ≥25 mL/min for women, and a screening random tHcy level ≥ 11 μmol/L for women, or ≥ 12 μmol/L for men. Patients will be stratified by clinic, and randomly assigned to treatment with a standard multivitamin containing a high dose combination of folic acid, vitamin B6, and vitamin B12, or an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12. All patients will receive standard clinical management for traditional CVD risk factor reduction. The study is designed to recruit 4000 patients (2000 in each group; 30%-35% in each group will have diabetes) over a 2-year period for ***83 to 87% power to detect a 19.0 to 20.0 % treatment effect during 5-years of follow-up.*** Preceded by a careful chart review, study eligibility is further determined in conjunction with a routine renal transplant clinic visit, with the addition of random tHcy and creatinine determinations. Appropriately processed and stored EDTA plasma and serum aliquots will be shipped to the central lab for tHcy and creatinine analysis each week. Only women with a random tHcy level ≥ 11 μmol/L, or men with a random tHcy level ≥ 12 μmol/L, as well as a serum creatinine based GFR ≥ 30 mL/min for men and ≥25 mL/min for women, will be eligible to be randomized. All data required for randomization will be made available to the clinical sites within ≤ 2-3 weeks of a potential participant's screening visit. The baseline/randomization examination requires: informed consent; medical history & detailed current medication review; intake of folic acid, vitamin B12, and vitamin B6 from supplements; basic physical activity data collection; random blood collection for tHcy, folate, vitamin B12, pyridoxal 5'-phosphate (PLP), lipid profile, creatinine, & glucose determinations. Patients will be stratified by clinic, and randomly assigned to receive a daily multivitamin devoid of folic acid, vitamin B12, or vitamin B6, or a multivitamin containing, in addition to other standard multivitamins, a high dose of folic acid, vitamin B6, and vitamin B12. ***Follow-up clinic visits each 12-months for evaluation*** will include general medical histories focusing on hospitalizations, emergency room , and physician's office visits; full medication inventories; intake of folic acid, vitamin B12, and vitamin B6 from supplements; pill counts; and blood tests. In addition, questionnaires regarding hospitalizations, & intake of folic acid, vitamin B12, and vitamin B6 from supplements, will be administered ***at 6-month intervals after each of these clinic visits, during telephone follow-up. Follow-up continues until death or a common end date of a minimum of 4.5- years after the last participant is randomized***. ***Follow-up for events is expected to continue through July 31, 2011 or until death. Study exit visits are planned to be conducted between August 1, 2011 and October 31, 2011.*** For the primary analysis of the primary pooled CVD endpoint, participants with allograft failure requiring initiation/re-initiation of chronic maintenance dialysis will be censored at 3-months post-dialysis. A secondary analysis of the same endpoint will be performed without censoring. Data analysis will be performed on the basis of original randomization (intention to treat) using the log-rank test of difference in survival-without-endpoint curves.

I. STUDY HYPOTHESES

Patients with chronic renal disease occupy the highest risk stratum for subsequent arteriosclerotic cardiovascular disease (CVD) events (1). The excess risk of CVD in chronic renal disease is due in part to a higher prevalence of established arteriosclerotic risk factors, including older age, hypertension, diabetes, dyslipidemia, and physical inactivity (1). However, unique renal insufficiency/"uremia"-related risk factors likely also contribute to this excess CVD risk (1). Prominent among these unique risk factors in the chronic renal disease population are elevated levels of the putatively atherothrombotic sulfur amino acid homocysteine (2). Homozygous genetic disorders (i.e., the "homocystinurias" [3-5]) resulting in marked hyperhomocysteinemia (total homocysteine levels of 100 to 500 μmol/L) are clearly associated with precocious atherothrombotic events (6), and total homocysteine (tHcy)–lowering treatment appears to reduce the incidence of such outcomes among these patients (6,7). In addition, pooled data from prospective observational studies suggest that mild to moderate hyperhomocysteinemia (tHcy levels of 12 to 99 μmol/L[8]) may also be a significant risk factor for arteriosclerotic CVD among general populations of men and women (9). However, randomized, controlled clinical trial data confirming these reported associations are unavailable (10). Moreover, the impact of cereal grain flour fortification with folic acid (10,11) on plasma tHcy levels within the general population may obfuscate the results from any such trials conducted in the United States. Chronic renal disease patients, ***including renal transplant recipients***, have an excess prevalence of mild to moderate hyperhomocysteinemia, which has been independently linked to their development of CVD outcomes in recent prospective observational studies (11-15).

Hypothesis: Lowering tHcy levels in patients with chronic renal disease will reduce their excess incidence of arteriosclerotic CVD outcomes.

Renal transplant recipients comprise a unique subpopulation for testing this tenable hypothesis within the overall chronic renal disease population, given:

A) the high rate of de novo and recurrent cardiovascular disease outcomes in these patients (1);

B) their excess prevalence of hyperhomocysteinemia in the era of folic acid fortified cereal grain flour, which *contrasts with all other* potential target populations with normal renal function (16);

C) the ability to safely and successfully "normalize" their tHcy levels with combined folic acid, vitamin B12, and vitamin B6 treatment (17,18), which differs dramatically from patients with true end-stage renal disease (19)

D) that renal transplant recipients (RTR) are a highly motivated group of patients (20) treated almost exclusively in large medical centers, which is conducive to overall recruitment into clinical trials, while minimizing sampling bias, and greatly enhancing follow-up for endpoint ascertainment; centralized care & follow-up of RTR stands in stark contrast to the diffuse care of patients with chronic renal insufficiency who have not yet reached end-stage renal disease (21)

E) overall "conditions" in the renal transplant population (renal impairment, mild-to-moderate hyperhomocysteinemia which can be normalized by B-vitamin supplements, and excess CVD outcomes) are representative of the larger population of patients with chronic renal insufficiency who have not yet reached end-stage renal disease (2,17,18).

<u>We are performing a randomized, controlled trial to test the following primary hypothesis:</u>

(I) Treatment with a high dose combination of folic acid, vitamin B6, and vitamin B12 will reduce the rate of pooled arteriosclerotic CVD outcomes (i.e., pooled occurrence of non-fatal and fatal arteriosclerotic outcomes, including coronary heart, cerebrovascular, and

peripheral vascular disease events = primary outcome), relative to treatment with an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12, among chronic, stable renal transplant recipients

We will also examine the following *secondary hypotheses* identified *a priori*:

(I) **Treatment with a high dose combination of folic acid, vitamin B6, and vitamin B12 will reduce the rate of total mortality relative to treatment with an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12, among chronic, stable renal transplant recipients**

(II) **Treatment with a high dose combination of folic acid, vitamin B6, and vitamin B12 *among chronic, stable renal transplant recipients with baseline diabetes, specifically*, will reduce their rate of pooled arteriosclerotic CVD outcomes (i.e., pooled occurrence of non-fatal and fatal arteriosclerotic outcomes, including coronary heart, cerebrovascular, and peripheral vascular disease events = primary outcome), relative to treatment with an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12**

(III) **Treatment with a high dose combination of folic acid, vitamin B6, and vitamin B12 will reduce the rate of decline in (creatinine-based estimates of) renal function, or the rate of graft failure requiring initiation of chronic dialysis, relative to treatment with an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12, among chronic, stable renal transplant recipients**

II. BACKGROUND AND SIGNIFICANCE

II.A. Chronic Renal Transplantation: A "Model" for Chronic Renal Insufficiency.

The overall chronic renal disease population comprises four subpopulations encompassing various stages, treatment modalities, and treatment settings. These include chronic renal insufficiency, defined as a reduction in glomerular filtration rate (GFR) due to chronic renal disease, generally manifest as an elevated serum creatinine; end-stage renal disease (ESRD) treated by hemodialysis, ESRD treated by peritoneal dialysis, and renal transplant recipients [RTRs] (1). RTRs were considered to have chronic renal disease because, typically, GFR is reduced and declines progressively over time. Although the most common cause of progressive renal function decline in RTRs is chronic rejection, a number of non-immunologic factors have been shown to be associated with chronic rejection, and commonly, such factors are identical to those associated with progressive renal disease in native kidneys (1).

II. B. Arteriosclerotic Cardiovascular Disease in Renal Transplant Recipients.

Table 1. Arteriosclerotic cardiovascular disease incidence after renal transplantation (from ref.22)

	Post-transplant incidence*		Expected**
	Patients without CVD pre-transplantation (%)	All Patients (%)	CVD Incidence (%)
1. Angina	6.4	10.3	2.3
2. Myocardial infarction	6.4	7.8	1.3
3. Transient ischemic attacks	3.7	4.5	-
4. Thrombotic strokes	3.3	3.7	0.6
5. Peripheral vascular disease	2.6	3.0	-
6. Coronary heart disease (1&2)	11.0	15.1	3.4
7. Cerebrovascular disease (3&4)	6.0	7.3	-
8. "Total " CVD (1&2&3&4&5)	15.8	21.3	4.7

* based upon a mean of 46 ± 36 months of follow-up; **Framingham data (23)

Arteriosclerotic cardiovascular disease (CVD) is the most common cause of death after renal transplantation (1,22), as well as a major source of morbidity (1,22). Renal transplant recipients (RTRs) experience at least twofold increases in arteriosclerotic CVD mortality (1,22), and fourfold increases in pooled non-fatal and fatal CVD incidence (1,22) see Table 1 above), relative to population-based estimates. Established arteriosclerotic risk factors such as age, sex, cigarette smoking, diabetes, hypertension, and dyslipidemia do not account adequately for this excess risk (1,22). Furthermore, management of dyslipidemia and hypertension in this patient population may be complicated by immunosuppressive medication interactions, and residual renal insufficiency, or renal vascular disease (24). Accordingly, there is a compelling need to identify and safely manage other putative CVD risk factors contributing to the excess occurrence of CVD among RTRs. Given these considerations, elevated plasma tHcy is an excellent candidate risk factor because tHcy-lowering can be achieved safely, relatively rapidly, and inexpensively, with B-vitamin intervention.

II.C. Determinants of Homocysteine Levels, and the Prevalence and Etiology of Hyperhomocysteinemia.

II.C. 1. Determinants of Homocysteine Levels, and the Prevalence and Etiology of Hyperhomocysteinemia in General Populations:

Approximately 70-80% of circulating plasma/serum tHcy is bound to large proteins (e.g., albumin) (8), the remainder consisting of a "free" acid-soluble fraction, i.e. reduced Hcy (<1%), homocysteine disulfide, and the predominant non protein-bound forms, homocysteine-mixed disulfides (8). Folate, pyridoxal 5'-phosphate (PLP or "active" vitamin B6), and vitamin B12, are the main vitamin co-factors / substrates for homocysteine metabolism. Vitamin B-12 and folate play critical roles in the remethylation of homocysteine to methionine (25). Betaine (trimethylglycine) is another substrate that participates in the remethylation of homocysteine to methionine via a B12 / folate-independent reaction (25). Vitamin B6 (as PLP), conversely, has a minor role in the remethylation pathway, but is crucial for the irreversible transsulfuration of homocysteine to cystathionine, as well as the subsequent hydrolysis of cystathionine to cysteine and alpha-ketobutyrate (25). Consistent with this underlying biochemistry, population-based data indicate that intake and plasma status of folate, vitamin B6, and vitamin B12 are important

determinants of tHcy levels (26). Mild, subclinical inherited defects in the key remethylation or transsulfuration pathway enzymes, alone or via interactions with B-vitamin status, may also influence tHcy levels in general populations (25,27). Selhub and Miller (25) have hypothesized that two distinct forms of hyperhomocysteinemia can result when normal S-adenosylmethionine (SAM)-regulated partitioning of homocysteine between the remethylation and transsulfuration pathways is disrupted. Impairment of the remethylation pathway due primarily (on a population basis) to inadequate status of folate or vitamin B12 results in hyperhomocysteinemia under fasting conditions. Conversely, impairment of the transsulfuration pathway is associated with normal or only very mildly elevated tHcy levels under fasting conditions, but substantial elevations following a methionine load. Both animal model findings (28), and clinical observations from humans (25) support this hypothesis. Indeed, a randomized, placebo-controlled 2x2 factorial designed tHcy-lowering intervention study recently demonstrated that B6 treatment independently reduced the 2-hour post-methionine load increase in tHcy levels among stable renal transplant recipients (17).

Creatinine (29) and albumin (30) are two additional, independent determinants of tHcy levels in general populations, unrelated to B-vitamin status. The generation of s-adenosylhomocysteine from s-adenosylmethionine is coupled to creatine-creatinine synthesis (31), which likely accounts for the direct association observed between creatinine and fasting tHcy levels in persons with normative renal function (29). As noted earlier, 70-80% of serum/plasma tHcy is protein-bound, most likely to albumin (8) which may account for the direct relationship between albumin and tHcy levels found in the general population (30).

Severe cases of hyperhomocysteinemia, as in homocystinuria, may be due to rare homozygous defects in genes encoding for enzymes involved in either homocysteine remethylation or transsulfuration. The classic form of such a disorder is that caused by homozygosity for a defective gene encoding for cystathionine beta synthase (CBS), a condition in which fasting plasma total homocysteine concentrations can be as high as 400-500 μmol/L (3,7). Homozygous defects of other genes that lead to similar elevations in plasma homocysteine concentration include those encoding for methylenetetrahydrofolate reductase (MTHFR) (4), or for any of the enzymes which participate in the synthesis of methylated vitamin B12 (5). (also see Table 2.)

II.C. 2. Determinants of Homocysteine Levels, and the Prevalence and Etiology of Hyperhomocysteinemia in Chronic Renal Transplantation/Chronic Renal Insufficiency.

Nine independent studies (32) reported between 1981 and 1999, provided data on free or tHcy levels in stable RTR. We recently provided controlled findings describing an increased prevalence of fasting hyperhomocysteinemia in RTR (33). In addition, our study provided the initial documentation of an apparent excess prevalence of post-methionine load hyperhomocysteinemia (matched odds ratio 6.9), and combined fasting and post-methionine load hyperhomocysteinemia (matched odds ratio 18.0) in renal transplant recipients, relative to age and sex-matched population-based controls with normative renal function (also see Table 3).

In an early (i.e., pre-cyclosporine/tacrolimus era) study of n=27 stable renal transplant recipients, Wilcken and colleagues (34) reported a significant association between creatinine and cysteine-homocysteine mixed disulfide within a range of serum creatinine, consistent with mild to moderately impaired renal function (i.e., serum creatinine of ~ 100-500 μmol/L). Consistent with these data, we found that renal function may be a particularly crucial determinant of tHcy levels in renal transplant recipients, both under fasting conditions (35), and post-methionine loading (33). Although Arnadottir and colleagues (36) have suggested that cyclosporine use exerts an "independent" influence on fasting tHcy levels in these patients, both matched analyses, and multivariable regression modeling of data from a total of over 500 RTR (35,37,38), have revealed that cyclosporine use is not an independent

determinant of tHcy levels, after appropriate adjustment for renal function indices (in particular), age, and sex. Recently, we performed an additional analysis which further validates these previously published findings (39). We measured fasting plasma tHcy, folate, pyridoxal 5'-phosphate (PLP), and B12 concentrations, in addition to serum creatinine and albumin concentrations, in 86 chronic, stable renal transplant recipients, and 238 patients with chronic renal insufficiency. The two patient groups had serum creatinine levels encompassing equivalent total ranges (i.e., renal transplant recipients = 53.0 to 371.3 μmol/L [0.6 to 4.2 mg/dL]; CRI = 61.9 to 362.4μmol/L [0.7 to 4.1 mg/dL]), with identical geometric means (renal transplant recipients = chronic renal insufficiency = 150.3 μmol/L [1.7 mg/dL]). Geometric mean tHcy levels did not differ between the groups in either unadjusted analyses (renal transplant recipients = 15.0 μmol/L; chronic renal insufficiency = 14.9 μmol/L, P = 0.899), or by general linear modeling with analysis of covariance adjusted for the major determinants of tHcy levels, i.e., age, sex, B-vitamin status, albumin, and creatinine (renal transplant recipients = 15.6 μmol/L; chronic renal insufficiency = 14.6 μmol/L, P= 0.173). As anticipated, renal function, gauged as a simple creatinine measurement, was the major independent determinant of plasma tHcy concentrations, accounting for ~ 80% to 90% of the total variability in tHcy predicted by the full model (i.e., full model R^2) containing, in addition to creatinine, the seven other potential explanatory variables. Finally, although unadjusted correlations between fasting plasma tHcy and folate levels among RTRs have been reported (34,36,37), multivariable modeling (35) has revealed that the independent strength of this association is minor, relative to a simple creatinine-based estimate of renal function.

It has been convincingly demonstrated that normal urinary excretion of homocysteine is trivial (40), and plasma elimination of homocysteine in ESRD is grossly retarded (40). However, a consensus statement from The Second International Conference on Homocysteine Metabolism (Nijmegen, The Netherlands, April, 1998; Drs. AG Bostom and CD van Steuhower, co-authors) concluded that at present, the ultimate etiology of the mild hyperhomocysteinemia so consistently noted in renal insufficiency (2,39) (including renal transplantation [2,32,39]) and ESRD (2), remains unexplained. Despite in vitro studies demonstrating renal tubular metabolism of homocysteine (41,42), and rat model evidence of significant in vivo renal homocysteine metabolism (43,44), non-significant *mean* human renal arteriovenous differences for (total and non-protein bound) homocysteine were recently reported (45).These findings (45) have rekindled a search for "uremia-induced" extrarenal (46), presumptively, hepatic defects in homocysteine metabolism. It should be noted, however, that mild decrements in glomerular filtration rate (GFR), *encompassing clearly non-uremic ranges of GFR*, determined either by direct measurement (47-49), or using a sensitive surrogate like cystatin C (50-52), are strongly and independently associated with (linear) increases in fasting tHcy levels.

Table 2. Range of Plasma Total Homocysteine Levels in Homocystinuria, End-Stage Renal Disease, Chronic Renal Insufficiency /Chronic Renal Transplantation, and the General Population (from ref. 32)

Group	10^{th} to 90^{th} Percentile Range of Plasma Total Homocysteine (μmol/L)
Homocystinuria	50-300
End-Stage Renal Disease	12-39
Chronic Renal Insufficiency*	9-25
Chronic Renal Transplantation*	9-25
Normal Renal Function, Population-Based Controls**	6-12

*chronic renal insufficiency patients receiving no immunosuppressive drugs, and chronic renal transplant recipients on standard immunosuppressive therapy, with equivalent renal function
**current era of folic acid fortified cereal grain flour

Table 3. Prevalence of Mild Fasting and/or Post-Methionine Load Hyperhomocysteinemia: Chronic, Stable Renal Transplant Recipients Versus Matched Population-Based Controls (from ref. 33)

	Renal Transplant Recipients	Age and Sex-Matched (2:1) Controls*
N	29	58
Total homocysteine levels:		
Fasting > 14.2 μmol/L, %	59**	9
Post-load increase > 26.1 μmol/L, %	32**	9
Fasting > 14.2 μmol/L *and* Post-load increase > 26.1 μmol/L, %	32**	2

*free of clinical renal disease, and with serum creatinine ≤ 1.5 mg/dL
** P< 0.001, from matched chi-square

II.D. Homocysteine and Arteriosclerosis: Epidemiological Evidence from Prospective Studies.

II.D.1. The Natural History of Cystathionine Beta Synthase Deficiency.
Through their painstaking characterization of the natural history of children and young adults with cystathionine beta synthase deficiency, Mudd and colleagues (6), first highlighted the potential link between marked hyperhomocysteinemia (i.e., equivalent to total homocysteine levels of 100 to 450 μmol/L by current assays), and premature atherothrombotic sequelae. In addition, these investigators provided the initial evidence that treatments designed to lower the markedly elevated total homocysteine levels observed in cystathionine beta synthase deficiency, appeared to reduce atherothrombotic event rates in this patient population.

II.D.2. Prospective Data from General Populations.
A consistent, but not unequivocal body of evidence has emerged during the past decade from prospective cohort studies of adult populations suggesting that even mild elevations in total homocysteine levels might confer an increased risk for cardiovascular disease outcomes (9). Ueland, Refsum, Beresford, and Vollset (9) recently performed a meta-analysis of 14 prospective studies of the relationship between baseline total homocysteine levels and (primarily) coronary heart disease outcomes in population-based cohorts, reported through the end of 1999. Most of the studies evaluated by these authors characterized the association between total homocysteine levels and risk of (primarily) coronary heart disease, upon adjustment for age, smoking, blood pressure, and serum cholesterol. Several studies further adjusted for body mass index, diabetes, and physical activity. For all studies, the authors calculated or estimated the risk per 5 μmol/L change in total homocysteine concentration. Nine of the 14 studies provided information specific to men and six provided information specific to women. Within the 4 studies that reported results for each sex separately, the pooled relative risk estimates for men, and the pooled relative risk estimates women, did not differ. Accordingly, the results for men and women from these studies were combined. Each of the 14 studies contributed one relative risk estimate, and pooling these, the aggregate relative risk estimate (from a total of 2786 cases) per 5 μmol/L change in total homocysteine concentration was 1.20 (95% CI = 1.14-1.25).

II.D.3. Prospective Data from Populations with Chronic Renal Disease.
Intractable survivorship effects resulting from the excess yearly mortality in ESRD (1), and the failure to establish whether or not arteriosclerotic outcomes antedated the development of ESRD, renders hazardous any inference about tHcy-CVD associations suggested by retrospective studies of patients treated by either maintenance dialysis or renal transplantation. The potential relationship between

hyperhomocysteinemia and arteriosclerotic outcomes in both chronic dialysis or RTR populations requires more rigorous validation via prospective observational studies, and ultimately, clinical tHcy-lowering intervention trials. Reported findings from each of the three published prospective studies of pre-dialysis, or dialysis-dependent ESRD populations (12-14), as well as the pooled analysis of these data (2), have revealed a linear trend for increased CVD risk, i.e., per μmol/L increase or across quantiles of tHcy. The prospective data reported by Ducloux et al (15) from chronic RTR, have also demonstrated a continuous relationship between tHcy levels and CVD risk, although, as expected, the greatest relative risk, was confined to the uppermost distribution. Specifically, Ducloux et al (15) have reported that among N= 207 RTR [age: mean ± SD = 48 ± 14 years old] operationally defined as "chronic and stable" (i.e., transplant duration > 6-months; no evidence of acute rejection; serum creatinine concentration < 4.5 mg/d), there were a total of 30 new CVD events (cumulative incidence = 14.5%), after a mean follow-up of 19.7 ± 4.4 months. Using multivariable-adjusted (i.e., for age, sex, prior CVD, creatinine, cyclosporine use, smoking, hypertension, diabetes, dyslipidemia, and tHcy) proportional hazards modeling, only age, serum creatinine, and fasting tHcy levels were independently predictive of CVD events during follow-up. *Each one μmol/L increase in tHcy was associated with a 6-7% increase in the risk for developing CVD* (i.e., multivariable-adjusted hazards ratio = 1.06 to 1.07, 95% confidence interval = 1.04-1.09; $P<0.001$).

II.E. Homocysteine and Arteriosclerosis: Experimental Evidence.

The pathologic mechanisms by which homocysteine promotes arteriosclerosis remain unclear. Experimental data (2,32) support a range of possibilities, including endothelial cell injury, enhanced low density lipoprotein oxidation, increased thromboxane-mediated platelet aggregation, inhibition of cell surface thrombomodulin expression and protein C activation, enhancement of lipoprotein (a)-fibrin binding, and promotion of smooth muscle cell proliferation. The in vivo relevance of findings from such experimental studies, however, has been seriously questioned (53) due to their lack of specificity to Hcy versus other much more abundant plasma thiols, including cysteine, and the use of grossly supraphysiologic concentrations or non-physiologic forms (i.e., D-L as opposed to L) of reduced Hcy. The data of Mansoor and colleagues (54) provide the background appropriate for adequate understanding of the specific criticism regarding grossly supraphysiologic concentrations. These investigators assessed concentrations of reduced Hcy across the widest possible spectrum of tHcy concentrations. Their data revealed that at tHcy concentrations of up to 100 μmol/L, levels of reduced Hcy accounted for only 1% or less (i.e., < 1 μmol/L) of plasma tHcy. When tHcy exceeded 100 μmol/L, reduced Hcy began to rise exponentially, likely due to saturation of plasma protein-binding sites (54). However, the highest reduced Hcy value these authors documented was in a subject with homozygous homocystinuria who had a tHcy > 350 μmol/L, but a reduced Hcy of < 100 μmol/L (54). When juxtaposed to the concentrations of reduced Hcy used in experimental studies (2,32), i.e., 1000 to 10,000 μmol/L, the findings of Mansoor and colleagues (54) illustrate the very dubious clinical relevance of these published data. In contrast, physiologic models of mild, dietary-induced hyperhomocysteinemia (i.e., tHcy ≤ 15 μmol/L) causing subclinical or frank atherothrombotic sequelae have recently been described in minipigs (55) and cynomolgus monkeys (56). Follow-up investigations employing these models may elucidate the in vivo relevance of the putative pathologic mechanisms outlined above.

II.F. Treatment of Hyperhomocysteinemia.

II.F.1. The Cystathionine Beta Synthase Deficiency "Experience".

The severe hyperhomocysteinemia (i.e., tHcy levels of 100-400 μmol/L; see Table 2) found in homozygous cystathionine beta synthase (CBS) deficiency has been treated with methionine restriction

and supraphysiologic doses of vitamin B-6, vitamin B-12, folate, and betaine (3,6,7). Such treatment lowers Hcy levels, and more importantly, appears to reduce the incidence of atherothrombotic events and mortality in these patients (3,6,7). Management of this severe form of hyperhomocysteinemia is the paradigm for treatment of the more common mild to intermediate forms of hyperhomocysteinemia. With the exception of methionine restriction, all the major therapeutic approaches to lowering Hcy attempted in homocystinuria, have been applied to populations with moderate hyperhomocysteinemia, including patients with chronic renal disease.

II.F.2. Treatment of ESRD Patients:

In dialysis-dependent/"dialysis-imminent" ESRD patients, folic acid, or reduced folate-based B-vitamin regimens, including folic acid/reduced folates at doses of 5-60 mg/day, B12 at up to 1 mg/day, and B6 at up to 100 mg/day, may lower fasting tHcy levels by up to 50%, but over 90% of treated subjects continue to exhibit mild to moderate hyperhomocysteinemia (2,57,58). *Moreover, in ESRD patients receiving standard of care daily multivitamins which contain 1 mg/day of folic acid, carefully controlled analyses have revealed that there is little or no further tHcy-lowering benefit of adding even grossly pharmacological doses of folic acid to this baseline supplementation regimen (59).*

II.F.3. Treatment of Renal Transplant Recipients:

Open label findings from RTR with much milder decrements in renal function (32) have suggested that these patients, in contrast, *are much less refractory* to high dose folic acid-based tHcy-lowering supplementation. We performed a block randomized, placebo-controlled two by two factorial study of n=29 clinically stable RTR (17) demonstrating that, in contrast to what we observed in their ESRD counterparts undergoing maintenance dialysis, the mild hyperhomocysteinemia in the RTRs proved very amenable to high dose combination B-vitamin therapy (folic acid 5.0 mg/day, vitamin B6 50 mg/day, and vitamin B12 0.4 mg/day). Treated patients experienced mean reductions of fasting and post-methionine load tHcy levels of ~ 25% after only 6-weeks, with 75% achieving "normalization" of their tHcy levels (17). In a subsequent investigation (18), we found that a standard US multivitamin dose of folic acid (i.e., 0.4 mg/day) provided clearly suboptimal tHcy-lowering efficacy relative to a supraphysiological dose (2.4 mg/day), in chronic, stable RTR. We have also demonstrated (19) that in comparison to renal transplant recipients with equivalent baseline tHcy levels, the mild hyperhomocysteinemia of ESRD patients undergoing maintenance hemodialysis is much more refractory to tHcy-lowering B-vitamin treatment regimens featuring *even greater* supraphysiological amounts of folic acid, or the reduced folate, L-5-methyltetrahydrofolate. Specifically, we compared the relative responsiveness of (n=10) RTR and (n=39) hemodialysis [HD] patients with equivalent baseline total homocysteine (tHcy) levels [i.e., RTR range= 14.2- 23.6 μmol/L; HD range= 14.4- 24.9 μmol/L] to 12-weeks of tHcy-lowering treatment. The RTR received 2.4 mg/day of FA, 50 mg/day of vitamin B6, and 0.4 mg/day of vitamin B12, while the HD patients received 15 mg/day of FA or an equimolar amount (17 mg/day) of the reduced folate, L-5-methyltetrahydrofolate, in addition to 50 mg/day of vitamin B6, and 1.0 mg/day of vitamin B12. The mean percent (%) reductions (± 95% confidence interval) in tHcy were: RTR=28.1% (16.2-40.0%); HD=12.1% (6.6-17.7%), P=0.027 for comparison of between groups differences by analysis of covariance adjusted for baseline tHcy levels. Moreover, 5/10 (50.0%) of the RTR versus only 2/39 (5.1%) of the HD patients had final on-treatment tHcy levels < 12 μmol/L, P=0.002 for comparison of between groups differences by Fisher's exact test. We concluded (19) that relative to RTR with comparable baseline tHcy levels, the mild hyperhomocysteinemia of maintenance HD patients is much more refractory to tHcy-lowering B-vitamin treatment regimens featuring supraphysiological amounts of folic acid, or the reduced folate, L-5-methyltetrahydrofolate. Accordingly, RTR are a preferable target population for controlled clinical trials testing the hypothesis

that tHcy-lowering B-vitamin intervention may reduce arteriosclerotic CVD event rates in patients with chronic renal disease (60).

II.G. Clinical Trials Testing the "Homocysteine Hypothesis" Against A Background of Folic Acid Fortification of Cereal Grain Flour.

The US Food and Drug Administration (FDA) published a regulation in early 1996 (61) that all enriched flour breads, rice, pasta, cornmeal, and other cereal grain products would be required to contain 140 μg of folic acid per 100 g by January 1998. A similar enriched flour fortification initiative has been under way in Canada (62). The goal of these fortification policies was to increase intake of folate by women of childbearing age to reduce the risk of neural tube defects. Cereal grain flour products fortified with 140 μg folic acid per 100g flour began appearing voluntarily in the United States after March, 1996 (10). We have demonstrated the profound effect that this fortification policy has had on the prevalence of both low folate status (i.e., a > 90% decline in the prevalence of plasma folate levels < 3 ng/mL), and mild hyperhomocysteinemia (a decline of ~ 50% in the prevalence of fasting plasma total homocysteine levels > 13 μmol/L) among chronic non-users of vitamin supplements in the population-based Framingham Offspring Study (10). The powerful impact of fortification in another region of the US was subsequently highlighted by crude time trend analyses of serum folate status in the enormous Kaiser Permanente Health Maintenance Organization database (63). Moreover, data just made available from the National Health and Nutrition Examination Survey (NHANES) III and NHANES 1999 (64) have confirmed the dramatic impact of this fortification policy on serum and red cell folate status among a representative sample of the entire US population.

Large randomized, controlled trials of total homocysteine lowering for the potential reduction of cardiovascular disease outcomes are ongoing in the United States and Canada (The Vitamin Intervention for Stroke Prevention [VISP], Women's Antioxidant Cardiovascular Disease [WACS], and Heart Outcomes Prevention Evaluation [HOPE-2] studies; see ref. 65 and Table 4). The dramatic impact of implemented policies to fortify cereal grain flour products with folic acid may reduce the statistical power of these trials. All three trials assume the active treatment groups will achieve the same mean total homocysteine lowering treatment effects reported in the absence of the background effect of folic acid fortified cereal grain flour. We re-examined those assumptions using data from total homocysteine lowering treatment efficacy studies conducted in populations with cardiovascular disease in the United States and Canada, exposed to folic acid fortified cereal grain flour products (66,67). These data reveal that VISP, HOPE-2, and WACS will likely achieve only about 20-25% of their projected mean total homocysteine lowering treatment effects (reductions of 1.0-1.5, versus 4.0-6.0 μmol/L). As a result, all three trials would be substantially underpowered to test their specific total homocysteine lowering hypotheses identified *a priori* (see Tables 4. & 5.). In contrast, renal transplant recipients exhibit a persistent excess prevalence of hyperhomocysteinemia in the era of fortification (16), while remaining very responsive to supraphysiological dose folic acid-based supplementation, achieving mean reductions in their total homocysteine levels of 5.0-6.0 μmol/L (18). Thus, unlike other high cardiovascular disease risk populations with normal renal function who are impacted profoundly by fortification efforts, renal transplant recipients are uniquely suited for a controlled trial of the "homocysteine hypothesis" (60,68).

Table 4. Design Features of Three North American Trials of Total Homocysteine Lowering for Cardiovascular Disease Outcome Prevention (from ref. 65)

Study [Reference(s)]	Start Date	Study Population	Main Outcome(s)	Treatment Regimen (per day oral doses)	Sample Size
Vitamins in Stroke Prevention [VISP; refs. 3,14]	1998	Patients with non-disabling stroke	Recurrent stroke	Folic acid 2.5 mg + B6 25 mg + B12 0.4 mg, vs. Folic acid 0.02 mg + B6 0.2 mg + B12 0.06 mg	3600*
Women's Antioxidant Cardiovascular Disease Study [WACS; ref. 3]	1998	Patients with (primarily) coronary artery disease, or multiple CVD risk factors	Pooled arteriosclerotic CVD outcomes	Folic acid 2.5 mg + B6 50 mg + B12 1 mg, vs. placebo	5449**
Heart Outcomes Prevention Evaluation [HOPE-2; ref. 3]	1999	Patients with (primarily) coronary artery disease, or diabetes and at least one other CVD risk factor	Pooled arteriosclerotic CVD outcomes	Folic acid 5 mg + B6 50 mg + B12 1 mg, vs. placebo	5000*

*Projected; recruitment ongoing; ** Recruitment completed short of projected goal of 8000

Table 5. Potential impact of fortification on mean plasma total homocysteine lowering effects and statistical power in VISP, WACS, HOPE-2, and renal transplant recipient (FAVORIT) trial (from ref. 68)

Trial	Difference in mean total homocysteine (μmol/L), treated vs. placebo group	Corresponding percent reduction in primary outcome rate (%)[c]	Corresponding Power[d,e]
VISP	- 5.0[a]	25.0%	≥ 95.0%
	-1.5[b]	7.5%	≤ 20.0%
WACS	- 4.0[a]	20.0%	≥ 95.0%
	-1.0[b]	5.0%	≤ 20.0%
HOPE-2	- 4.0[a]	20.0%	≥ 95.0%
	-1.0[b]	5.0%	≤ 20.0%
FAVORIT	- 5.5 [b]	27.5%	≥ 95.0%

[a]Pre-fortification era projection; [b]Likely effect post-fortification based on references 8,9,17;

[c]Assumes each μmol/L decrease in mean plasma tHcy results in a 5% reduction in the CVD outcome rate of interest;

[d]Based on sample sizes listed in Table 1, placebo group event rates standardized to 20.0% for each trial, and a two-tailed alpha of 0.05;

[e]Based on total renal transplant recipient trial population of 4000, 2000 receiving active treatment, and 2000 receiving placebo treatment

III. EXPERIMENTAL DESIGN AND METHODS

III.A. Study Population.

III.A.1.Eligibility.

III.A.1.1. Definition of Chronic, Stable Post-Transplant Renal Function.

The primary fundamental eligibility criterion is that patients evidence chronic, clinically stable renal function post-transplantation. Kidney-pancreas transplant recipients with stable renal graft function will also be eligible for study participation, as will recipients of bone marrow transplants. Recipients of any other organ transplants, such as liver, heart or lung, are not eligible. Stable renal function will be ascertained by careful chart review establishing that the patient's current graft has been functioning for at least six-months post-transplantation, patients are not in the midst of treatments for acute rejection, and a Cockcroft-Gault serum creatinine based estimates (69) of GFR are ≥ 30 mL/min for men and ≥25 mL/min for women. At pre-randomization "eligibility" visits (i.e., routine renal transplant clinic visits), random serum will be obtained, aliquoted, stored at -80 degrees C, and sent on dry ice by overnight courier (in weekly batches) for serum creatinine determinations to be made by the central laboratory, to confirm that the individual patient's current creatinine based estimates of GFR are ≥ 30 mL/min for men and ≥25 mL/min for women. Within 2-weeks, the central lab will provide the results of these creatinine analyses to the Data Center for transmission to the clinical site.

III.A.1.2. Definition and Determination of Mildly Elevated Total Homocysteine (tHcy) Level.

The second fundamental eligibility criterion is a random plasma tHcy level ≥12.0 μmol/L for men, or ≥11.0 μmol/L for women. Plasma tHcy will be determined by a modification of the method of Araki and Sako (70). The method is reliable and accurate (see protocol appendix). In advance (~2-3 months) of their regularly scheduled clinic visits, potentially eligible patients will be contacted. Those currently using vitamin supplements will be asked to abstain from multivitamin, B-complex, or specific individual vitamin supplements containing folic acid, vitamin B6, or vitamin B12, for at least 4-weeks prior to their examination. Informed consent for this specific purpose (i.e., abstention from usual vitamin use) will be obtained. As indicated above for serum creatinine, during these pre-randomization "eligibility" visits (i.e., routine renal transplant clinic visits), random EDTA plasma will obtained, aliquoted, stored at -80 degrees C, and sent on dry ice by overnight courier (in weekly batches) for plasma tHcy determinations to be made by the central laboratory, to confirm that the individual patient's tHcy levels are ≥ 11.0 μmo/L for women or ≥ 12.0 μmol/L for men. Within 2-weeks, the central lab will provide the results of these tHcy analyses to the Data Center for transmission to the clinical site.

III.A.1.3. Additional Inclusion Criteria.

In addition, inclusion into the trial requires the following:

1. Age 35 to 75 years at time of randomization
2. Cognitive function adequate for patient to give accurate information
3. Geographically accessible for follow-up
4. Informed consent
5. Adequate transportation facilities

III.A.1.4. Exclusion Criteria.

Presence of:

1. Cancer, end-stage congestive heart failure, liver, or pulmonary disease, progressive HIV or other chronic wasting illness, which in the opinion of the study physician, would limit the life expectancy of the patient to less than two

years or prevent evaluation of recurrent or de novo CVD; Other conditions that prevent reliable participation in the study, such as refractory depression, severe cognitive impairment, or alcoholism or other substance abuse
2. Pregnant or lactating women or women of childbearing potential not practicing birth control.
3. Participation in another clinical trial specifically involving CVD risk factor management
4. Inability to be randomized within 120 days of screening.
5. Less than 3-months post acute myocardial infarction, or stroke, or less than 3-months post coronary artery, renal artery or lower extremity artery PTCA, or lower extremity amputation
6. Less than 6-months post coronary artery bypass graft surgery, abdominal aortic aneurysm repair surgery, or carotid endarterectomy.

III.B. Recruitment and Follow-Up Schedule.

III.B.1. Screening and Recruitment.
Initially, twenty large academic renal transplant centers across North America will be participating in the proposed study. Additional transplant centers will join the study in 2005. By database review, potentially eligible patients under the care of each transplant center will be contacted for consent to be screened for the trial during their next regularly scheduled clinic visit. They will be informed that this screening process will entail abstaining from multivitamin, B-complex, or specific individual vitamin supplements containing folic acid, vitamin B6, or vitamin B12, for at least 4-weeks prior to their examination, and having two to six additional tubes of random blood drawn. The clinical evaluation must be sufficient to establish eligibility and rule out any exclusion criteria. Each center must also determine that the patient is able to be randomized within 120 days of screening. After the 120 day timeframe the patient would have to be rescreened. Distance from the clinic and extended vacations must be carefully reviewed. The local team, consisting of the nephrologist or transplant surgeon, principal investigator, and the study coordinator, is responsible for verifying that the patient meets all the eligibility criteria except the tHcy and creatinine levels/creatinine-based GFR estimates (to be available within 2-3 weeks). The patient coordinator completes the study Recruitment Log daily, continuing until recruitment is completed. [The Manual of Procedures provides additional details relevant to the enrollment and randomization methods; treatment; procurement and dispensing of study vitamins.]

III.B.1.1. Simple Screening Visit.
This screening method is used for participants who, if eligible, will return to the transplant clinic within 120 days of screening for the clinic randomization visit. Completing the informed consent process is the first activity. The clinical evaluation must be sufficient to establish eligibility and rule out any exclusion criteria. Only two tubes of random blood are drawn. A sample of participants will provide one additional tube of blood for blind replicate quality control assessments.

III.B.1.2. Screening/Baseline Combination Visit.
For eligible participants who will be randomized over the telephone, much of the baseline data collection coincides with screening to become a screening/baseline combination visit. Completing the informed consent process is the first activity. The clinical evaluation must be sufficient to establish eligibility and rule out any exclusion criteria. Six tubes of random blood are drawn, and a clean catch urine specimen is collected. A sample of participants will provide one additional tube of blood and/or a

urine specimen for blind replicate quality control assessments. Blood pressure, height, weight, relevant medical history, regular medication use, and personal identifying information are obtained.

III.B.2. Enrollment and Randomization Methods.

The study nephrologist/transplant surgeon assesses the patient's eligibility and suitability and gives approval prior to enrollment in the screening/eligibility phase. Thereafter, this physician and/or the nurse coordinator discuss with the patient and family member(s) the nature, importance, potential benefits and risks, and duration of the study. The patient is informed that he/she may or may not qualify for the next phase of the study, the randomized clinical trial, depending on the results of the blood tests. The patient is informed that he or she is at liberty to refuse participation or to withdraw at any time. If the patient agrees, properly witnessed informed consent is obtained, and the patient is scheduled for a randomization clinic visit or telephone contact within 3-4 weeks. The Central Lab screening data are forwarded to the Data Center which in turn (typically within 48-hours) notifies the clinical center that a patient has met the tHcy & creatinine eligibility criteria, and the patient can be randomized pending confirmation of ongoing eligibility at the time of the actual randomization visit or telephone contact . Interested persons who do not meet laboratory eligibility criteria may be "re-screened" at their next scheduled routine clinic visit.

Eligible subjects will be randomized in a double blind manner to one of two treatment groups: multivitamins containing a high dose combination of folic acid, vitamin B6, and vitamin B12, or an identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12. The permuted block method for the random allocation will be used. At least three different block sizes will be used, with the order of blocks determined randomly. Patients will be stratified by clinical center, only. To implement this procedure, a sequence of treatment assignments will be computer generated for each stratum. These assignments will be merged with the sequence of post-randomization participant ID number. To reduce the possibility of randomization error and improve security, a microcomputer based randomization procedure is proposed. The data management system provided to each clinic will include the clinic's sequence of vitamin supply assignments in an encrypted file. After entering the required participant identifying and pre-randomization information the software will confirm the participant's eligibility and permanently assign the medication supply number from the clinic's sequence. Only then would the vitamin number to be used be displayed to the clinic staff. Using this approach, the vitamin supplies need not be assigned sequentially, eliminating any possibility of clinic staff manipulating the order of treatment assignments.

III.B.3. Baseline and Follow-up Evaluations.

III.B.3.1. Overview. (See also Table 6)

Prior to obtaining screening bloods at a routine clinic examination, the clinic database and individual patient clinic records/charts will have been thoroughly reviewed by the site PI and nurse coordinator to assure that potential participants meet basic eligibility criteria, and to establish the absence of obvious exclusion criteria. Potential participants will be then be contacted by telephone and informed that the screening process will entail abstaining from multivitamin, B-complex, or specific individual vitamin supplements containing folic acid, vitamin B6, or vitamin B12, for at least 4-weeks prior to their scheduled clinic examination, and having additional tubes of random blood drawn. Patients who have provided informed consent for the study at the screening exam, and are subsequently confirmed eligible for the study by their laboratory tests (i.e., for tHcy and creatinine/estimated GFR), will be randomized at either a study renal transplant clinic visit or over the telephone pending confirmation of continued eligibility.

III.B.3.1.1. Clinic Randomization Visit.
Participants who completed the Simple Screening Visit need to return to the renal transplant clinic for a study visit if they are lab-eligible to have final eligibility confirmed and be randomized. Entry examination at time of randomization will include an updated history focused on *relevant* (i.e., specific to CVD and/or renal disease, diabetes, or the major exclusion criteria, namely any condition that would limit the life expectancy of the patient to less than two years or prevent evaluation of recurrent or de novo CVD, and pregnancy) intercurrent hospitalizations, emergency room, or physician's office visits; intake of folic acid (in particular), vitamin B12, and vitamin B6. Blood pressure, height, weight, relevant medical history, regular medication use, brief, focused queries regarding physical activity, cereal consumption, and dietary supplement use, and personal identifying information are obtained. Four tubes of random blood are drawn, and a clean catch urine specimen is collected. A sample of participants will provide one additional tube of blood and/or a urine specimen for blind replicate quality control assessments.

III.B.3.1.2. Telephone Randomization Contact.
Participants who completed the Screening/Baseline Combination Visit and meet laboratory eligibility criteria will be contacted by telephone to determine final eligibility for the study. Eligibility will be assessed for all non-laboratory criteria as of the time of the telephone contact, so participants will be queried about *relevant* (i.e., specific to CVD and/or renal disease, diabetes, all intercurrent hospitalizations, emergency room, or physician's office visits; intake of folic acid (in particular), vitamin B12, and vitamin B6; age; transplant history; major illnesses or conditions that would limit participation in the study; and if applicable, child-bearing potential.

III.B.3.1.3. Follow-up Evaluations.
After randomization, patients return periodically for (yearly) clinic visits or are contacted by phone (intercurrent 6-months; see Table 6.). Yearly clinic follow-up largely mirrors the clinic randomization visit. A sample of participants will provide one additional blood collection tube or urine specimen for blind replicate quality control assessments. Telephone follow-up includes history (focused on intercurrent *hospitalizations*), determination of study vitamin compliance (i.e., pill counts by patient), and assessment of intake of folic acid (in particular), vitamin B12, and vitamin B6 from non-study vitamin supplement capsules/tablets. Also, during each follow-up telephone and clinic visit, study participants will be asked to report *possible* adverse reactions to the vitamins. These limited queries will focus on pruritus, urticaria, and gastrointestinal disturbances. Telephone interviews and clinic visits are alternated every ***six*** months ***until the end of follow-up*** , or until the occurrence of death. Participants who develop dialysis-dependent ESRD will be followed until their **first** primary outcome occurs, after which mortality surveillance sufficient to distinguish CVD from non-CVD death continues until the end of the follow-up period for that participant. The scheduled date of the visit or phone interview is determined by the date of randomization, not by the date of the previous contact. All interviews and clinic exams are to be made within ten days of the scheduled date. If the follow-up schedule must be changed due to illness, geographic relocation or extended vacation, procedures are followed to document the change in schedule. If a patient misses an appointment s/he or her/his family will be contacted by the patient coordinator by phone or mail to inquire about a possible recurrent or de novo CVD event, return to chronic dialysis, or death.

Table 6. Visit Schedule. Required exams and procedures by month on study. RC= regular renal transplant clinic visit; SC= study renal transplant clinic visit* (*coordinated with RC visits to as great an extent as possible; "SC/RC"); P= telephone "visit"; tHcy= total homocysteine; PLP= pyridoxal 5'-phosphate

Procedure	RC Simple Screening	RC Screening/ Baseline	SC Base-Line/ Randomization 0	P Randomization	Follow-up P	Follow-up SC/ RC
(Prior) Chart Review	X	X				
Random tHcy & creatinine	X	X				
Random bloods for tHcy, folate/B12, PLP, Lipid profile, creatinine, glucose, fructosamine, & archiving		X	X			X
Clinic Exam & Medical History		X	X			X
Medical History (abbreviated)					X	
Medication Inventory		X	X			X
Focused Adverse Reactions Survey					X	X
Focused Surveys: a) Intake of supplemental folic acid, vit. B6, & vit. B12 b) Physical Activity		X X	X X			X X
Final Eligibility Check			X	X		
Pill count						X

III.B.3.2. Central Laboratories Blood and Urine Studies.

All study bloods will be drawn random with recording of time since last consumption of any liquids or solids other than water. For screening eligibility, venous blood (7 ml in EDTA; 7 mL without anticoagulant) from samples properly collected, handled, and separated, will be aliquoted into EDTA plasma, serum, and buffy coat aliquots, and cryopreserved (in the –80 degree C freezers provided by the study) at each clinical site. Screening EDTA and serum aliquots will be regularly (~weekly) shipped on dry ice by overnight courier for central determination of tHcy and creatinine levels at the Vitamin Bioavailability (i.e., tHcy), and Nutrition Evaluation Laboratories (creatinine), respectively, within The Jean Mayer USDA Human Nutrition Research Center on Aging in Boston, MA. For the baseline and yearly follow-up visits thereafter, venous blood (20 ml in EDTA, i.e., two 10 mL tubes; 10 mL without anticoagulant; 7 mL citrate) will be drawn. For ALL study visits, the EDTA and citrate containing tubes will be immediately placed on wet ice and centrifuged (in a refrigerated centrifuge) within 3 hours at 2800 rpm for 15 minutes at 4 degrees C. The EDTA and citrate plasma will be separated, immediately refrigerated, and aliquoted in special screw capped cryopreservation tubes. The whole blood without anticoagulant will be allowed to clot for 15-20 minutes, and the serum separated, immediately refrigerated, and aliquoted in special screw capped cryopreservation tubes. For patients randomized into

the intervention phase, specific visits will also include obtaining and saving buffy coat aliquots from the EDTA tube, once the plasma has been aliquoted. In addition, spot urine samples will be obtained at the randomization, and each of the follow-up visits, immediately refrigerated, and then aliquoted into 1.5 mL aliquots for long-term storage at – 80 degrees C. All specimens requiring centrifugation should be placed in the centrifuge within 3 hours of collection. All processing should be completed and the aliquots placed in the –80 degree C freezer within 4 hours of specimen collection. EDTA plasma, serum, citrate plasma, buffy coat, and urine aliquots cryopreserved at -80 degrees C, will be utilized as follows:

1) for tHcy (EDTA plasma, 0.5 mL aliquot)
2) for folate, PLP, and vitamin B12 (EDTA plasma, 0.5 mL aliquot)
3) for creatinine & glucose (serum 0.5 mL aliquot)
4) for total cholesterol, HDL-cholesterol, direct-LDL, and triglycerides (serum, 1.0 mL aliquot)
5) for fructosamine (serum, 0.5 mL aliquot)
6) for albumin/creatinine ratio* (urine, 1.0 mL aliquot) *(pending ancillary study support)
7) for additional ancillary studies/archiving (multiple 0.5 mL aliquots of EDTA and citrate plasma, buffy coat, serum, & 1.5 mL aliquots of urine)

III.B.3.3. CVD Risk Factor Surveillance.

All randomized patients are continued on their usual post-transplant medical/surgical and traditional CVD risk factor management. The risk factors predisposing toward recurrent or de novo clinical arteriosclerotic CVD are well recognized and include cigarette smoking, both diastolic and systolic hypertension, sedentary lifestyle/obesity, diabetes mellitus, elevated serum LDL cholesterol and/or reduced HDL-cholesterol. These risk factors will be assessed and recorded, but only pre-determined "alert" values for blood pressure and body weight/body mass index, as well as *any* ongoing cigarette smoking, will be reported to the patient's primary care physician, for further specific evaluation and treatment of individual patients. There are formal American Society of Nephrology guidelines for managing cardiovascular disease risk factors in renal transplant recipients. These guidelines will be copied and distributed to each site Principal Investigator. While plasma and serum will be obtained at each clinic visit for the purposes of the study, these specimens will be aliquoted, stored, banked, and batch-analyzed, and will *not* be available for clinical purposes. To reduce participant burden, study clinic physicians and nurse coordinators will synchronize usual clinical care blood draws with study blood draws to the greatest extent possible. Follow-up of these specific clinical test results will be via standard mechanisms in place at each center.

CVD risk factor surveillance will include:

1. *Hypertension*. At each visit, blood pressure will be measured twice, 10 to 15 minutes apart, with the patient sitting, and use of antihypertensive medications recorded.
2. *Obesity*. The patient's body weight and height will be recorded at each clinic visit.
3. *Dyslipidemia*. Sera will be stored from each clinic visit for analysis of total cholesterol, HDL cholesterol, LDL-cholesterol, and triglycerides, and use of lipid lowering medications recorded.
4. *Diabetes mellitus*. Sera will be stored from each clinic visit for analysis of fructosamine and glucose, and use of insulin preparations and/or oral anti-diabetic agents recorded.
5. *Cigarette smoking*. Patients will be questioned about cigarette smoking at each clinic visit, and the average number of cigarettes smoked per day, recorded.
6. *Sedentary lifestyle*. Responses to brief, focused queries will be obtained from each participant at the randomization/baseline visit, and yearly until the end of the study.

III.B.3.4. Assessment of Supplemental Intake of Folic Acid, and Vitamins B6 and B12.
Supplemental intake of folic acid, vitamin B6, and vitamin B12, will be assessed at each of the annual clinic visits (i.e., from vitamin capsules/tablets, as well as heavily fortified cereals & liquid/powdered supplements), and the semi-annual telephone interviews (from vitamin capsules/tablets, only) to obtain data on any changing levels of non-study vitamin supplementation.

III.C. Intervention.

III.C.1. Treatment Protocol.
All patients are continued on usual post-transplant general medical and CVD risk factor management as determined by their treating physician. *Half the randomized patients will receive multivitamins containing high doses of folic acid, vitamins B6 & B12, and the other half will receive* an *identical multivitamin containing no folic acid, and Estimated Average Requirement (EAR) amounts of vitamin B6 and vitamin B12.* ***To address any concerns about randomizing study participants to a treatment tablet devoid of folic acid,*** we analyzed whole food intake (i.e., *exclusive* of dietary supplements) of total folate (expressed as Dietary Folate Equivalents [DFEs], see below) in n=46 RTRs (35) using the Willett Food Frequency Questionnaire (71). Based on the recent Institute of Medicine [IOM] Report (72), in order to account for the increased bioavailability of folic acid (i.e., "synthetic" folate, vs. naturally occurring food folate), each food must be partitioned into the component that is synthetic folate (i.e., "added" to whole foods), and that which is naturally occurring. The amount that is synthetic must be multiplied by a factor of 1.7 to account for its increased bioavailability. This "corrected" synthetic value must then be added to the naturally-occurring amount in order to express the total amount of folate as "Dietary Folate Equivalents" [DFEs], in each food. Based upon recently published data (73), we determined the amount of synthetic folate (i.e., folic acid) the RTRs surveyed obtained from specific fortified breakfast cereal products (35), ***as well as an estimate of the synthetic folate they obtained as a result of "generic" fortification of cereal grain flour as per the Food and Drug Administration mandate (10,73).*** If any patient becomes pregnant during the study they will be encouraged to take standard prenatal vitamin supplements regardless of study treatment arm.

Table 7. Whole food, non-supplement intake of dietary folate equivalents in n=46 chronic, stable renal transplant recipients (RTR), updated to reflect impact of flour fortification using latest Framingham Study estimates

Estimated Average Requirement* (i.e., recommended population 50th percentile)	RTR 50th percentile	Lowest single RTR value
320 mcg	646 mcg	346 mcg

*Institute of Medicine recommendation

In summary, *the absolute lowest* daily whole food intakes of DFEs among the surveyed RTRs was above the estimated average requirements for this micronutrient (72).

The composition of the two multivitamins (see Table 8 below) has been adapted from Nephro-Vite, a supplement developed specifically for end-stage renal disease. The composition of the vitamins for the two vitamin treatment arms are listed in Table 8). Patients will be instructed to take one tablet a day for the length of the study. Study tablets will be re-supplied each 12-months in lots of 400.

Table 8. – "Active and "Placebo" Vitamin Formulation Contents

COMPONENT	**"Active" Formulation**	**"Placebo" Formulation**
Vitamin B6 (Pyridoxine HCl)*	**50 mg**	*1.4 mg*
Folic acid *; **	**5.0 mg**	**0.0 mg****
Vitamin B12*	**1.0 mg**	*2.0 mcg*
Vitamin B1 (Thiamine HNO3)*	**1.5 mg**	**1.5 mg**
Vitamin B2 (Riboflavin)*	**1.5 mg**	**1.5 mg**
Vitamin C (Ascorbic Acid)*	*60 mg*	*60 mg*
d-Biotin***	**300 mcg**	**300 mcg**
Niacinamide*	**20 mg**	**20 mg**
Pantothenic Acid (Calcium Pantothenate)***	**10 mg**	**10 mg**

***All values, (i.e., placebo or "active" formulations), at or above the Estimated Average Requirement (EAR)**
**Fortification of all enriched cereal grain flour provides an average of 340 mcg/d folate to non-supplement users
*** All values, (i.e., placebo or "active" formulations), at or above the Average Intake (AI), since EAR is unknown

III.C.2. Procurement and Dispensing of Study Vitamins.
The vitamin supplements will be supplied by Pamlab, L.L.C. (Covington, LA), being manufactured, stored, and distributed by Anabolic Laboratories. However, Pamlab, L.L.C. is designated as the Vitamin Distribution Center (VDC). The responsibilities of the VDC include: procurement of the necessary materials, coding and labeling of the packaged vitamins, and storage and distribution of the finished products, with appropriate quality controls at each stage. Because the study is double-blind, the vitamins will be dispensed with identical appearing tablets, bottles, closures, and external seals. Matching of the two dosages will be pre-tested but is not likely to be a problem because both tablets will contain active ingredients, although weight and specific gravity of the tablets could differ.

III.D. Trial Conduct.

III.D.1. Determination of Endpoints.

III.D.1.1. Overview of Composite Primary Endpoint.
The primary end point is recurrent or de novo arteriosclerotic cardiovascular disease (CVD), defined as the occurrence of non-fatal or fatal arteriosclerotic outcomes, including coronary heart, cerebrovascular, and peripheral vascular disease events. ***These outcomes will include: CVD death or nonfatal major arteriosclerotic events, specifically: myocardial infarction, resuscitated sudden death, coronary artery***

revascularization, stroke, and requirement for an invasive procedure for peripheral or renovascular disease (i.e., angioplasty/stenting, endarterectomy, aneurysm repair, or lower extremity amputation for an arteriosclerotic complication). All relevant medical history and records will be obtained, and any potential arteriosclerotic CVD outcomes will be validated according to standardized definitions of the outcomes, with putative endpoints reviewed on an ongoing basis by the Clinical Endpoints Center.

III.D.1.2. Adjudicated Endpoints.

Events to be adjudicated are as follows:
Death
Myocardial Infarction
Stroke
Resuscitated Sudden Death

Patients will be followed for the above events from the time of randomization until the date patient follow-up ends, or death, whichever occurs first. The primary outcome will be the first occurrence of FAVORIT-defined "pooled CVD", with (statistical) censoring at 3-months post-dialysis (in addition to censoring at end of study follow-up period, or death). For the primary pooled CVD endpoint, renal transplant recipients who become dialysis-dependent prior to experiencing a primary outcome, will be followed until their first primary outcome occurs, after which mortality surveillance continues until the end of the follow-up period for that participant. Clinical sites will be notified as early as possible when such primary outcomes have been adjudicated, at which point only mortality surveillance sufficient to determine CVD vs. non-CVD death will be required. Renal transplant recipients who become dialysis-dependent after experiencing a primary outcome will undergo only mortality surveillance sufficient to determine CVD vs. non-CVD death, until the end of the study. Again, the Data Center and Endpoints Core will work assiduously to inform the clinic about adjudicated (non-fatal) primary outcomes (in the "pre-dialysis" observation period), as early as possible.

III.D.1.2.1. Fatal Events: Cardiovascular Versus Non-Cardiovascular.
Death will be classified in two categories, cardiovascular or non-cardiovascular. All deaths will be assumed cardiovascular in nature unless a non-cardiovascular cause can be clearly shown.
Death will be classified in the following categories:

I. Arteriosclerotic Coronary Heart Disease

Acute Myocardial Infarction
Sudden Death
Non-Sudden Death
- Unwitnessed Death
- (Coronary) Procedural Death

II. Arteriosclerotic vascular disease, excluding coronary disease

Cerebrovascular disease, i.e., stroke
Aortic, mesenteric, renal vascular, or peripheral vascular disease
Procedural: death occurring during a hospitalization for or after a vascular procedure (i.e., carotid endarterectomy, abdominal aortic aneurysm repair), when the circumstances surrounding the death can be linked to a vascular procedure.

III. *Other Cardiovascular Disease (Non-Arteriosclerotic)*

Pulmonary Embolism
Endocarditis
Valvular Disease
Procedural
Other

IV. Non-Cardiovascular

Infectious
Malignancy
Pulmonary
Gastrointestinal
Accidental
Suicide
Diabetes
Renal
Other

V. Unknown

III.D.1.3. Unadjudicated Procedural Endpoints.

1. Coronary artery disease (i.e., undergoing coronary artery revascularization, either bypass surgery or angioplasty)
2. Lower extremity arterial disease (i.e., undergoing lower extremity arterial angioplasty or bypass surgery, or for severe disease [rest pain and/or gangrene], lower extremity amputation above the ankle).
3. Extracranial carotid arterial disease (i.e., undergoing carotid endarterectomy [CEA], or angioplasty).
4. Abdominal aortic aneurysm (i.e., undergoing abdominal aortic aneurysm [AAA] repair).
5. Renovascular disease (i.e., requiring renal artery revascularization, either bypass surgery or angioplasty)

III.D.1.4. Reporting of Death.

In the event of the death of a study patient all possible efforts will be made to obtain relevant records from the hospital or the patient's primary care physician, including death certificates, to determine cause of death. An Outcomes Documentation form is completed and entered into the data entry system as soon as possible. If the patient was admitted to a hospital during his/her final illness, a Hospitalization form is also completed. Since there may be considerable delay in obtaining the records needed to complete some of these forms, all deaths are to be reported to the DCC within three days of the date that the clinical center is notified of the event, regardless of availability of records. Mortality classification forms will be completed for all participants who die during the course of the study.

III.D.1.5. Graft Failure.

Graft failure will be defined as return to dialysis, and will be considered as a secondary outcome. Retransplantation, for the purposes of censoring, will **not** be considered a graft failure. Participants who develop dialysis-dependent graft failure will be followed until their first primary outcome occurs, after which mortality surveillance continues until the end of the follow-up period for that individual. Participants who have had a primary outcome prior to the initiation (re-initiation) of dialysis will have

mortality follow-up sufficient to distinguish CVD from non-CVD mortality until the end of the follow-up period for that participant. For the purpose of the primary analysis of the primary endpoint, participants with graft failure will contribute person-time until the first primary outcome or until three months *after* the resumption of dialysis, whichever is *earlier*. A secondary analysis of the primary endpoint will be done without censoring at three months post-graft failure. (see also earlier section **III.D.1.2.**)

III.D.2. Evaluation of Adverse Reactions.
Although we have found absolutely no placebo-controlled evidence of adverse reactions to doses (comparable to or greater than those for the proposed trial) of folic acid, vitamins B6 and B12 given to either maintenance dialysis or renal transplant patients, during follow-up telephone and clinic visits, study participants will be asked to report possible adverse reactions to the vitamins. These limited queries will focus on pruritus, urticaria, and gastrointestinal disturbances. Symptoms suggesting severe allergy are grounds for discontinuation of treatment. Mild gastrointestinal distress, presumably representing placebo effect, will be discussed with the patient. At each contact the patient will be asked about these possible adverse study vitamin reactions, and responses will be recorded on the appropriate forms. Patients required to discontinue study vitamin treatment or refusing to take it will not be removed from the study. Investigators will attempt to re-institute study vitamins in any patients who discontinue them. *Given the lack of serious side effects of multivitamins, emergency unblinding should rarely be required. In most cases, adverse events can be managed without knowledge of treatment assignment discontinuing study vitamins if appropriate. Clinical centers will be provided with the home phone numbers of two DCC staff members who will keep treatment assignment lists at home, in case unblinding is necessary.* Because the plasma tHcy and B-vitamin level results could unblind the investigators, these will be restricted to the DCC, and Data and Safety Monitoring Board.

III.D.3. Concomitant Medications and Vitamin Supplements.
Patients will be encouraged to use only the study multivitamins and not to take additional multivitamins. A special study brochure of instructions will be developed and distributed to patients. Use of all medications, and vitamin capsules/tablets, will be queried and recorded at each yearly clinic visit. Clinic visits will also include focused assessment of heavily fortified cereals & liquid/powdered supplements. During telephone follow-up, only vitamin/capsule use will be queried and recorded.

III.D.4. Masking.
Tablets for the high dose folic acid, vitamin B6, vitamin B12, and placebo folic acid, EAR dose vitamin B6, and EAR dose vitamin B12 arms, will be identical in size, shape, color, odor, and markings, consistent with the double-blind design of the protocol. The bottles, closures, seals, labels (except the code numbers) and markings for all packaging of study drugs will also be identical for the two kinds of tablets.

III.D.5. Monitoring Compliance.
At standard intervals (i.e., yearly clinic visits), plasma will be drawn for blind analysis of folate, vitamin B12 and PLP levels to assess compliance. Results will be available only to the Data Coordinating Center. In addition, at each clinic visit, the patient will bring study containers dispensed at the previous visit, and the patient coordinator will count the tablets remaining. Returned containers will be handled by the patient coordinator using the method to be provided by the VDC. Tablet counts and their variance from prediction will be recorded on the appropriate form. Visit adherence reports will also be monitored for each clinical center and overall, and reports will be distributed to the PIs, Operations Center, the Executive Committee, and the Data and Safety Monitoring Board. Steps will be taken within the trial structure to assist a clinic with lagging performance. Reasons for missed visits and phone calls will be

recorded. Non-compliance with study vitamins, operationally defined as < 75% compliant by pill count, will be tracked site by site, and routinely reported to the Executive Committee. Study coordinators will encourage non-compliant patients to improve their compliance by focusing, for example, on synchronizing study vitamin intake with their usual immunosuppressive drug intake (drugs which renal transplant recipients are typically religious about taking). Because statistical analysis will be based upon intention to treat, non-compliant patients will be encouraged to adhere to protocol but will not be removed from the study, because in all probability, this will occur equally in both groups.

III.D.6. Data Collection Instruments.
Examples of data forms for the pre-screening chart review/phone contact, screening visit, randomization visit, and follow-up telephone and clinic visit examinations are listed below, and actual samples are available on the secure FAVORIT website (http://www.cscc.unc.edu/favorit/) :

1. Pre-Screening Form (PRS)
2. Screening Form (SCR)
3. Screening Phlebotomy Forms (SPC and SPP)
4. Randomization Visit Forms (REL, RPC, MSR, PUF, PHC, and PHP)
5. Follow-Up Contact Form (FUP)
6. Vitamin Distribution Log (VDL)
7. Hospitalization Report Form (HOS)
8. Outcomes Documentation Form (OUT)

III.D.7. Data Management.
A PC-based distributed data management system (DMS) will be used for this study. The data management system will be implemented using Visual FoxPro Version 6.0 and installed on computers in each Clinical Center. This system will provide all of the capabilities required for research data management, including: data transfer, data entry, data validation, database updating, database closure, data retrieval, data inventory, security and confidentiality, and archiving, and in addition will support randomization.

Each Clinical Center will be responsible for entering and transferring the data it collects. The DMS will be installed on at least one computer per site, with the capacity for multiple desktop or laptop computers per site for data collection. The clinical center staff will use the DMS to enter screening data and eligibility data, run an algorithm to determine eligibility, and for each eligible patient, the DMS will issue a random treatment assignment. Follow-up data will also be entered at the clinical sites into the DMS.

One primary computer at each Clinical Center will have the capability to randomize participants and will host the main integrated database system. Centers with multiple computers for data collection will consolidate the data on these systems into the main database on the designated primary computer. The main database system will be used to generate reports for the Clinical Center and will be used to produce data transfer files for transfer to the Data Coordinating Center (DCC) at the Collaborative Studies Coordinating Center (CSCC) at the University of North Carolina.

III.D.7.1 Data Entry and Validation.
Direct data entry, where data initially are entered on the screen without having completed a paper form first, will be available at each center. Direct data entry eliminates the time-consuming and error prone process of keying from paper forms. Paper versions of each data collection instrument will be available as backup in situations in which the computer systems are inaccessible for any reason. In addition, if

there are forms that are routinely collected on paper for convenience or another reason, then the data on these forms will subsequently be keyed at the Clinical Centers using the distributed data entry system. The data entry system will display data entry screens that closely resemble the paper data collection forms. The system will be menu driven, with context-sensitive help available at any time.

Each data field will be edited during entry. Values that fail a validation routine will cause a message to be displayed. The person entering the data will then have three options:

- To correct the value, in which case the new value will be validated as was the previous entry;
- To flag the value as questionable, in which case the system will generate a printed data query form to document the question, and for use in recording a resolution; or
- To confirm the value as known to be correct, overriding the validation routine.

The data entry system will flag each data value with a "status character" documenting the current validation status of the item (empty, skipped, questionable, clean, confirmed, etc.).

The DMS provided to the Clinical Centers will include the ability for each center to generate locally a variety of summary reports concerning the data completeness, outstanding questionable values, etc. It has been our experience that most study coordinators find such a capability valuable in permitting them to monitor the quality of their center's performance. This facilitates timely identification and resolution of problems in data collection and processing.

III.D.7.2 Data Transfer and Processing.
Data from the main database at each Clinical Center will be transferred electronically to the DCC on a regular schedule. The transfer files will be encrypted when created by the clinical center data management system. Upon receipt, data files are logged in and imported to the study's database that consolidates data from all of the sites and the central laboratories. On a regular basis, data will be retrieved from the consolidated database and SAS® datasets will be prepared for statistical analysis purposes.

III.D.7.3 Central Laboratory Data Management.
The central laboratories will prepare data files from their local data management systems in a standardized format and transfer these to the DCC on a regular schedule. Upon receipt at the DCC, these data files will be processed analogous to the clinical center data files.

III.D.7.4 Data Security.
The DMS will require entry of a valid user ID and password for use. Sensitive files will be encrypted. Regular back-ups that are stored apart from the DMS will be required.

All data transferred to the DCC will be stored, processed and analyzed within the CSCC office suite. At the CSCC, all access to office space containing data is controlled through locked doors. Visitors are screened by SCC staff and cannot move about without a CSCC escort. All office space is locked after working hours. Access to computer data is controlled by passwords released only to those CSCC personnel who use the files. In addition, critical data files are encrypted.

III.D.7.5 Data Reporting.

On a monthly basis, the DCC will prepare a study data report that provides clinic-specific and overall summaries of patients screened and randomized by month. Timeliness and completeness of follow-up contacts will also be reported. In addition, the DCC will routinely generate reports for the clinical sites and laboratories concerning data quality (missing or overdue forms, outstanding data queries, etc), and facilitate the timely review, correction and resolution of data quality issues at the clinical sites.

III.D.7.6 Public release of data.

If the study is required by NIH to release a version of data for public use or if the executive committee approves release of the data to an ancillary study, all personal identifiers will be removed. All federal recommendations for insuring anonymity of the data will be implemented. The CSCC has experience with distributing data files both to study investigators and to the public, and has in place a multilevel check to ensure that confidential identifiers are not released. This check is performed by different staff members at the time a data distribution is requested, again by the programmer preparing the file to be distributed, by the head of the programming staff who reviews all such programming, by the study manager, and by the Coordinating Center PI.

III.D.8. Study Communications and Monitoring.

The Operations Center will maintain current contact information on the study web page for all study staff from the clinical sites, central laboratories, Data Coordinating Center, and Operations Center. Methods of study communications will include e-mail, web-postings, telephone, fax, and regular mail.

III.D.8.1 Technical Support.

The clinics will have a liaison at the DCC who can be called for an immediate answer to an operational or data management question or for help in obtaining clarification of a particular situation. For questions of a clinical nature, the clinics have a liaison at the Operations Center who directs the question to the appropriate committee chairperson. For each clinic, a primary study coordinator is identified, and a principal transplant nephrologist or surgeon investigator is identified who has the overall responsibility for the recruitment of patients and management of the study at the center.

III.D.8.2 Site Visits.

Site visits will be made to individual participating centers by a clinic monitor from the DCC or Operations Center to observe patients during clinic visits and to compare data sent to the DCC to that in the hospital records to verify adherence to protocol. In addition, if recruitment falls below a certain level, appropriate personnel designated by the Steering Committee, such as a team consisting of a transplant nephrologist or surgeon from a highly productive center, can be sent to advise on recruitment strategies. Monthly status reports of data quality and participant follow-up are prepared and circulated. These are reviewed to determine which clinics may need to be visited.

III.D.9. Statistical Power Calculations and Analysis Plan.

III.D.9.1. Power Calculations.

The primary comparison will test the null hypothesis that there is no difference between treatment groups in overall event rates, versus the alternative that the rates differ, using a *two sided* test with alpha=.05. All randomized patients will be included in the primary analysis, according to the group to which they are randomized, whether or not they actually receive study treatment (i.e., an intention to treat analysis.) The power calculations are based on looking at whether or not each subject has a CVD

outcome in the course of the study, rather than on the time to such an outcome, using the conventional normal approximation to the binomial distribution. *This is a conservative approach since the planned primary analysis using the log-rank test to compare the survival curves provides slightly greater power*. Subjects within a stratum/treatment group combination are all assumed to have the same risk of experiencing a CVD outcome. We have assumed that there are two primary strata--subjects with a history of diabetes, including those with successful pancreas transplants (35%; see discussion below) and subjects without a prior history (65%). Those with a history of diabetes are assumed to be at higher risk of experiencing a CVD outcome in the course of the study than those without a prior history. However, in order to calculate power we needed to make some assumptions about how many subjects would leave their assigned treatment group and the effect on disease risk for these subjects. We have assumed that subjects who drop out of the group assigned to the multivitamin devoid of folic acid, and with EAR amounts of vitamins B6 & B12, have the same risk as those who remain in this group. That is, the multivitamin devoid of folic acid, and with EAR amounts of vitamins B6 & B12, has no effect on risk of CVD. Subjects assigned to this multivitamin group who purchase and consume the B-vitamins of interest, and subjects assigned to the group given a multivitamin containing high doses of folic acid, vitamins B6 & B12, who do not adhere to this treatment but purchase and consume B vitamins over the counter, are assumed to receive a lower dose of these vitamins than the subjects who adhere to treatment with the multivitamin containing high doses of folic acid, vitamins B6 & B12. These subjects experience just some fraction of the benefit provided by the multivitamins containing high doses of folic acid, vitamins B6 & B12. Subjects who drop out of the treatment group receiving multivitamins containing high doses of folic acid, vitamins B6 & B12 and do not consume any multivitamin supplements are assumed to have the same risk of disease as subjects assigned to the multivitamin devoid of folic acid, vitamins B6 & B12, who adhere to this treatment.

The power depends on the underlying rate of the event(s) of interest. The background section reviews the available published information (6-10). However, for the calculations described herein, we updated this previously published information (6-10) with specific estimates for CVD incidence rates more germane to FAVORIT, as kindly provided by the United States Renal Data System, USRDS (Drs. Shuling Li and Charles Herzog, unpublished information; for details, see Protocol Appendix 1). In brief, as of January 1, 1995, a cohort of 12, 358 renal transplant recipients with the following criteria was established: at least 6-months of stable graft function as of January 1, 1995; Cockcroft-Gault estimated creatinine clearance of at least 30 mL/min within 6-months forward in time after January 1, 1995; and age between 35 to 75 years old on January 1, 1995. Using a start date of January 1, 1995, five-year incidence densities for the pooled (first) occurrence of any of the following non-fatal or fatal CVD events were calculated: myocardial infarction; stroke (atherothrombotic or hemorrhagic); abdominal or thoracic aortic aneurysm repair; revascularization for coronary artery, carotid arterial, renal arterial, or lower extremity arterial disease; lower extremity amputation above the ankle. Censoring events included: 30-days after return to dialysis (*i.e., 10% of the sample over 5-years*); loss to follow-up; death; or the end of December 31, 1999. The demographics of this cohort are characterized in Table 9.

Table 9. Demographics Distribution (N=12,358)

Mean Age (years)	49
Gender	
Male (%)	61.22
Female (%)	38.78
Race	
White (%)	74.83
Black (%)	19.89
Native America (%)	1.27
Asian (%)	3.11
Others (%)	0.91
Primary Diagnosis	
Diabetes (%)	23.40
Hypertension (%)	16.09
Others (%)	60.51

The CVD incidence rates of this USRDS sample cohort, stratified by presence or absence of diabetes (i.e., diabetes as the putative cause of ESRD, only), are presented in Table 10.

Table 10. Combined events including acute MI, stroke, aneurysm, amputation, revascularization, and CVD death.

	Follow-up Time				
Primary Diagnosis	1-year	2-year	3-year	4-year	5-year
Non-DM (N=9466)	308 (3.25%)	584 (6.17%)	864 (9.13%)	1106 (11.68%)	1285 (13.57%)
DM (N=2892)	339 (11.72%)	599 (20.71%)	778 (26.9%)	913 (31.57%)	1018 (35.2%)
Overall (N=12,358)	647(5.24%)	1183(9.57%)	1642(13.29%)	2019(16.34%)	2303(18.64%)

Random sample surveys of the twenty proposed FAVORIT centers (see Table 13) revealed that ~**35% of those renal transplant recipients meeting basic eligibility criteria** (i.e., with respect to age, time since renal transplantation, and current creatinine clearance) **were diabetic** (i.e., currently undergoing treatment with insulin or oral anti-diabetic medications). Power calculations used the USRDS diabetic stratum- specific CVD rate (in Table 2, above), applied to a projected FAVORIT population whose prevalence of diabetes at randomization would be 35%. Based on the tHcy screening eligibility criterion, our published treatment data (18) and the prospective data of Ducloux et al (15), the active treatment will reduce tHcy levels by a mean of ~6 μmol/L, which *could* translate into a reduction in the CVD event rate of ~35-40%. However, we have made the following conservative final estimates, based on the germane USRDS sample event rates (above) for both the pooled CVD outcome of interest, and the development of dialysis-dependent ESRD: ***With a sample size of 4000, and with 5% of each treatment group assumed to take no vitamins, and 5% of each group assumed to instead take a standard over the counter vitamin preparation, power is calculated to be 83.0% to detect a 19% treatment effect, and 87% to detect a 20% treatment effect, i.e., either a 19.0% or 20.0% reduction in their pooled CVD***

event rate, for those assigned to the multivitamin containing high doses of folic acid, vitamins B6 & B12.

III.D.9.2. Analysis Plan.

The results of high dose folic acid, vitamin B6, & vitamin B12 combined with standard multivitamin supplementation and usual chronic post-transplant medical management and CVD risk reduction, will be compared to standard multivitamin supplementation devoid of folic acid, but containing EAR amounts of vitamins B6 and B12, and usual chronic post-transplant medical management and CVD risk reduction, taking into account both stratification and randomization strategies. This will be done by comparing the treatment groups with respect to the distribution of time from randomization to first event. A log rank test will be used for this comparison, the main test of the primary hypothesis of the study. For this main test, patients will be censored three-months after allograft failure requiring initiation/re-initiation of chronic maintenance dialysis. In a secondary analysis of the primary hypothesis, the analysis will be by intention-to-treat, with no censoring after allograft failure.

The Kaplan-Meier method will be used to estimate unadjusted treatment-specific survival curves and to test for differences at the various times. Proportional hazards models will also be used, if the assumptions are satisfied, to adjust for other variables, such as initial (and or serial /"time-dependent") blood pressure, cigarette smoking, diabetes (diabetic recipients of successful pancreas transplants will be classified as diabetic since they carry the accumulated effects of prior years of diabetes), levels of lipids/lipoproteins, and creatinine-based renal function. The same analysis methods will be applied to secondary outcomes (i.e., total mortality; pooled CVD outcomes within the diabetic stratum; and development of dialysis-dependent ESRD). Analysis will also be done by sex, age group, race and by tHcy levels at the randomization visit. Treatment group comparisons, for interim and final analyses, will be for primary events, and secondary events (i.e., death; development of ESRD). Planned sample sizes for the study are based only on the analysis of differences between treatment groups in the time to primary event both because that relates to the main hypothesis of interest and because it is not expected that the treatment will affect rates of noncardiovascular disease death. However, differences in any of the three types of analysis found to be significant by our interim 'stopping rule" will be reported to the Monitoring Board for consideration.

III.D.9.3. Stopping Rules.

The Data and Safety Monitoring Board (DSMB) will examine the data at several points in time to determine whether the study should be stopped. To assist them, comparison of endpoint probability curves will be made at periodic intervals. As quoted by Halperin et al. (74) from Canner, (75) "..decision making in clinical trials is complicated and often protracted..", thus, "..no single statistical rule or procedure can take the place of the well-reasoned consideration of all aspects of the data by a group of concerned, competent, and experienced persons with a wide range of scientific backgrounds and points of view." The investigators agree with Halperin et al that, "statistical analyses of the accumulating data play an important but not dominant role". The particular statistical techniques chosen to determine the advisability of early stopping are stochastically curtailed tests. The trial is terminated if, at semi-annual examination, it appears that conditional on current data, either it is likely the null hypothesis will be (I) rejected at the end of the study, or (II) it is highly likely that the null hypothesis will not be rejected. In monitoring for efficacy or harmful effects, a number of methods for the repeated analysis of accumulating data have been proposed and used. When considering the stopping of a trial in which efficacy of the experimental treatment is claimed, the method used for monitoring the trial should be conservative: the trial should be stopped only if the treatment is clearly

superior. The O'Brien-Fleming' type boundary provides such a conservative approach (76). When the number and/or timing of interim evaluations cannot be fixed in advance, a Lan-DeMets (77) type spending rule which approximates the O'Brien-Fleming boundary is often used (76). The DSMB has proposed two interim looks: at approximately one-third and at two-thirds of expected numbers of events. In order to provide the flexibility to adjust to DSMB requests for changes in this schedule, we will use a Lan-DeMets boundary (78). In addition, conditional power as proposed by Halperin (74) will be used in the decision to stop the trial if the difference between treatments is small. This method computes the conditional probability of rejecting the null hypothesis given a specific alternative and the data at the time of the analysis. If this probability is too small, one may choose to discontinue the trial. Computationally, we will use a generalization of the method of Lan and Wittes (78).

III.E. Study Timetable.

Table 11.

Months	Planning, including final protocol, operations manual, and training	Recruitment	Follow-Up	Phase-Out and Analysis
Months 0-6 (August 01-Jan 02)	X			
Months 7-12 (Feb 02-July 02)		X		
Months 13-18 (August 02-Jan 03)		X	X	
Months 19-24 (Feb 03-July 03)		X	X	
Months 25-30 (August 03-Jan 04)		X	X	
Months 31-36 (Feb 04-July 04)		X	X	
Months 37-48 (August 04-July 05)		X	X	
Months 49-60 (August 05-July 06)		X	X	
Months 61-66 (August 06-Jan 07)		X	X	
Months 67-123 (Feb 07-Oct 11)			X	
Months 124-127 (Nov 11-Feb 12)			X*	X

*Time period for Clinics to compile final hospitalization data and to complete all data checks.

III.F. Statement on the Use of Human Subjects in FAVORIT.

III.F.1. Basic Rationale.

Pooled observational studies suggest that mild to moderate hyperhomocysteinemia (tHcy levels of 12 to 99 μmol/L[8]) may be a significant risk factor for arteriosclerotic CVD among general populations of men and women (9). However, randomized, controlled clinical trial data confirming these reported associations are unavailable. Moreover, the impact of cereal grain flour fortification with folic acid (10,16) on plasma tHcy levels within the general population may obfuscate the results from any such trials conducted in the United States. Chronic renal disease patients have an excess prevalence of mild to moderate hyperhomocysteinemia, which has been independently linked to their development of CVD outcomes in recent prospective observational studies (11-15). Renal transplant recipients comprise a unique subpopulation for testing this tenable hypothesis within the overall chronic renal disease population, given: the high rate of de novo and recurrent cardiovascular disease outcomes in these patients (1,15,22); their excess prevalence of hyperhomocysteinemia in the era of folic acid fortified cereal grain flour, which *contrasts with all other* potential target populations with normal renal function (16); the ability to safely and successfully "normalize" their tHcy levels with combined folic acid, vitamin B12, and vitamin B6 treatment (17,18), which differs dramatically from patients with true end-stage renal disease (2,19,32); that renal transplant recipients (RTR) are a highly motivated group of patients (20) treated almost exclusively in large medical centers, which is conducive to overall recruitment into clinical trials, while minimizing sampling bias, and greatly enhancing follow-up for endpoint ascertainment; centralized care & follow-up of RTR stands in stark contrast to the diffuse care of patients with chronic renal insufficiency who have not yet reached end-stage renal disease (21); overall "conditions" in the renal transplant population (renal impairment, mild-to-moderate hyperhomocysteinemia which can be normalized by B-vitamin supplements, and excess CVD outcomes) are representative of the larger population of patients with chronic renal insufficiency who have not yet reached end-stage renal disease (1,2,32).

III.F.2. Study Population.

The subject population consists of renal transplant recipients, at least 6-months post-transplantation. Specific inclusion and exclusion criteria are described in the Research Design and Methods section. *The goal of the study is 4000 randomized patients, ranging in age from 35 to 75. Patients are not excluded on the basis of sex or race. Women and minority groups will be recruited actively in order to achieve balanced representation. Published data are inconclusive on significant differences of clinical or public health importance in intervention between men and women; and there are essentially no data on differences between racial/ethnic subgroups.* We have adopted strategies for selecting centers, and for patient recruitment that should assure recruitment of representative numbers of the relevant subgroups. We asked potential centers to provide information about the gender and racial/ethnic distribution of renal transplant recipients at their centers. Several of the centers have clinical populations with a proportion of non-white patients substantially in excess of the overall US population (University of Alabama-Birmingham [African American]; UCLA [Hispanic]; Hennepin County Medical Center and London Health Sciences Center [Native American]; Oregon Health Sciences Center [Asian]). *Our most recent survey data from the participating sites are consistent with the USRDS sample data in Table 14 below, with respect to the prevalence of men and women (i.e., we expect our study population to be* 40% *female). Moreover, given the ethnic composition of the centers noted above, we expect our overall study population to include at least the 25% non-whites observed in the relevant USRDS sample (see Table 12, below). All of the centers have participated in other clinical trials involving renal transplant recipients and their expertise in recruiting and retaining these patients will be utilized in the training of personnel during the final protocol development and field testing phase. Specific methods for*

recruitment and retention in minority populations will be covered and detailed in the Operations Manual, which will include race and gender information; will be monitored to determine if there are differences between these subgroups in the proportion of randomizations to the number eligible. Up-to-date reporting will be stressed to ensure early identification of problems so that appropriate measures can be taken.

Table 12. Demographics of USRDS Sample Meeting Basic FAVORIT Eligibility Criteria

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	White, not of Hispanic Origin	Other or Unknown	Total
Percents of USRDS* Total (N=12,358)	1.3%	3.1%	19.9%	74.8%	0.9	100%

*USRDS sample meeting basic FAVORIT eligibility criteria: 61.2% men; 38.8% women

III.F.3. Data to be Collected.
Medical history, demographic data, and data from specific tests and stored specimens (as detailed earlier) are collected on patients who are screened and likely to be eligible. Additional and follow-up data are collected only on patients with random tHcy levels ≥ 11 or 12 μmol/L and estimated GFRs ≥ 30 mL/min for men and ≥25 mL/min for women, who are randomized. Sources of the data include the patient himself, the patient's family, and the patient's medical records. The vast majority of the data collected are specifically for research purposes, and not for patient care. Those data collected specifically for study purposes and reimbursable by the study include: blood drawn at screening, randomization, and every 12 months for determination of tHcy, creatinine, lipids, and glucose. Potential DNA analyses to be performed will relate ***only*** to possible genetic causes of CVD or renal disease, and/or abnormalities of homocysteine metabolism.

III.F.4. Recruitment and Consent Procedures.
Following an extensive chart review, potentially eligible patients are recruited from the renal transplant centers where they receive their routine follow-up care. They sign an informed consent form for the entire study that allows for screening bloods to be drawn at a regular clinic visit, and complete the process to affirm that they meet study eligibility criteria. When the Central Laboratory notifies the center that specific patients have met the tHcy and estimated GFR eligibility criteria, these patients are eligible to participate in the intervention phase. At randomization, these patients are informed again of the purpose of the intervention phase of the study, the treatment alternative, the random manner of assignment to treatment, the need to be available for telephone follow-up and return clinic visits at regular intervals for questionnaires and study phlebotomy, and of their options to accept or refuse entry into the study.

III.F.5. Potential Risks.
Butterworth and Tamura (79) found no evidence of adverse effects when folic acid was administered at up to 15-60 mg/day. Bendich and Cohen (80) have reported that vitamin B6 at doses of up 200 mg/day for two to twenty years was not associated with peripheral neuropathy, or other deleterious effects.

Chronic (2 to 15+ years) oral or parenteral vitamin B12 at 1 mg/day has been given safely to elderly patients with pernicious anemia (81), and children with homocystinuria and methyl malonic aciduria due to inborn errors of metabolism (82). Concerns about masking (while not treating) cobalamin deficiency with supraphysiologic doses of folic acid given without concomitant vitamin B12 (81) will not be relevant to the proposed study as the 5.0 mg folic acid dose is always given in a tablet also containing 0.4 mg of vitamin B12 (81,83). Published data from short term placebo-controlled interventions in both maintenance dialysis (2,57,58) and renal transplant recipient patient populations (17,18) are in accord with these earlier findings. We believe the risks of the vitamin therapy proposed are so minimal as to be inconsequential. This is a particular advantage both for safety and cost effectiveness.

III.F.6. Protecting Against Potential Risks.

III.F.6.1. Protecting Against Potential Risks to Personnel.
Biological hazards associated with these investigations relate to personnel exposure to blood and blood-borne pathogens in clinical specimens. Accordingly, the following guidelines and standards will be adhered to in addressing health and safety concerns for laboratory and clinical research personnel. "Universal Blood and Body Fluid Precautions," as established by Centers for Disease Control, will be utilized to include personnel training, specimen handling, use of protective barriers, and waste management. Diagnostic laboratory specimens submitted to the Tufts USDA HNRCA Vitamin Bioavailability Laboratory will be packaged, labeled, and transported in a manner consistent with the National Committee on Laboratory Standards". In accordance with the Occupational Safety and Health Administration's final rule on transmission of blood-borne pathogens, a written 'exposure control plan' will be established that identifies personnel at risk for occupational exposure to blood and other potentially infectious materials and training and information on specific "engineering controls" to protect them against exposure will be provided, including hepatitis B vaccination and post-exposure evaluation and follow-up in the event of an exposure".

III.F.6.2. Protecting Against Potential Risks to Patients.
Confidentiality of patient computer data is protected by the use of passwords, data encryption and secure, limited access storage. The DCC has programs, policies and facilities in use at the present time to ensure the security and confidentiality of the data it manages.

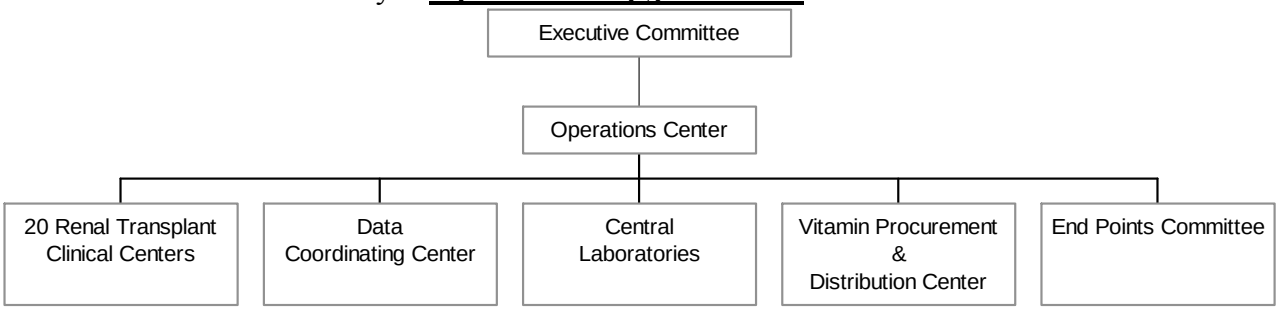
The DCC, and the NIH-appointed Data and Safety Monitoring Board play key roles in detecting any hazards the study may pose for its participants. Data are routinely collected and regularly monitored to watch for unusual mortality or morbidity associated with study-related procedures in each clinic. Timely reports will be made to the Monitoring Board. In addition, the SCC is responsible for calling the Board's attention to significant interim developments. Results for the different clinics are compared to identify the sources and causes of any remarkable deviations from the average performance. The Monitoring Board is responsible for advising early termination of the trial in the event that unexpectedly large treatment differences provide overwhelming evidence in favor of one intervention before the scheduled end of the trial. Early termination may also be considered if it becomes clear that the study is unlikely to be able to demonstrate a significant treatment difference. It will be left to the Monitoring Board to weigh the evidence and advise on early termination.

III.F.7. Risks versus Benefits.
There is little risk but potentially great benefit if the administration of high dose vitamin supplement proves to be a potent intervention for reducing the excess risk of arteriosclerotic CVD events in chronic, stable renal transplant recipients.

IV. TRIAL AND COMMITTEE ORGANIZATION

IV.A. Trial Organization.

Basic infrastructure of the study is ***depicted in the figure below:***



IV.A.1. Clinical Centers.

Listed below are the names of clinical centers willing to participate in the trial and their principal investigators, subject to final approval by the Executive Committee. Table 15 has a compilation of random chart review survey results completed in August/September 2001 to provide a hard estimate of the number of patients at each of these sites meeting basic FAVORIT eligibility criteria with respect to age, time since transplantation, and current creatinine clearance.

30 Primary Centers

University of Wisconsin- Madison	John Pirsch, MD
University of Alabama-Birmingham	Clifton Kew, MD
University of California-San Francisco	Deborah Adey, MD
Ohio State University	Todd Pesavento, MD
Cedars-Sinai Health System	Alice Peng,MD
University of Toronto	Edward Cole, MD
University of California-Los Angeles	Gabriel Danovitch, MD
Oregon Health Sciences University	Douglas Norman, MD
Medical College of Wisconsin	Barbara Bresnahan, MD
University of Maryland	Matthew Weir, MD
Washington University (St. Louis)	Matthew Koch, MD
University of Indiana	Muhammad Yaqub, MD
University of Michigan	Akinlolu Ojo, MD
Rhode Island Hospital/Lifespan	Andrew Bostom, MD
University of Iowa	Lawrence Hunsicker, MD
Albany Medical Center	David Conti, MD
Duke University	Stephen Smith, MD
Hennepin County Medical Center	Bertram Kasiske, MD
SUNY Health Science Center-Brooklyn	Mariana Markell, MD
London Health Sciences Center	Andrew House, MD
Northwestern University	Lorenzo Gallon, MD
University of Minnesota	Arthur Matas, MD
Mayo Clinic	Fernando Cosio, MD
Brigham and Women's Hospital	Ajay Singh, MD
Maine Medical Center	John Vella, MD
Banner Good Samaritan Transplant Services	Alfredo Fabrega, MD

Universidade Federal de Sao Paulo	Alvaro Pacheco-Silva, MD, PhD
Southern Illinois University	Tim O'Connor, MD
Drexel University	M.S. Anil Kumar, MD
East Carolina University	Paul Bolin, Jr., MD

Back-up Centers

Columbia Presbyterian	Mark Hardy, MD, and David Cohen, MD
University Hospitals of Cleveland	Donald Hricik, MD
Rush Presbyterian (Chicago)	Janis Orlowski, MD
Emory University	Carlos Zayas, MD

Table 13. Listing of renal transplantation centers agreeing to participate in proposed trial, with approximate numbers of eligible patients at each site.

Center	Approximate # patients meeting basic eligibility criteria & regularly attending center clinic	Approximate # of ***diabetic*** patients meeting basic eligibility criteria & regularly attending center clinic
University of Wisconsin-Madison	2340	1170
University of California-San Francisco	2380	770
University of Alabama-Birmingham	2014	836
Ohio State	1377	357
University of Pittsburgh *	1368	540
University of Toronto	910	260
University of California-Los Angeles	636	172
Oregon Health Sciences University	1075	301
Medical College of Wisconsin	648	207
University of Maryland	1806	903
Washington University-St. Louis	900	330
University of Indiana	770	210
University of Michigan	1244	526
Rhode Island Hospital/Lifespan	468	163
Albany Medical Center	483	224
University of Iowa	666	204
Duke University	570	278
SUNY-Downstate	264	92
London Health Sciences Center	482	176
Hennepin County Medical Center	664	160
TOTALS	20,365	7879

Lastly, 4-additional transplantation centers, (i.e., Columbia Presbyterian Medical Center; Rush Presbyterian; Emory University; and Municipal Hospitals of Cleveland) have agreed to be considered "back-up" centers in the event that one of the 20-selected centers cannot continue to participate.

IV.A.2. Operations Center.
Dr. Andrew G. Bostom, the Principal Investigator is the Project Director of the Operations Center. He has the overall responsibility for the study and chairs the Executive Committee. While the committee structure described below advises the Executive Committee in many areas related to the scientific conduct of the study, the Operations Center generally is the coordinating center for all the clinical and administrative activities of the trial. The Executive Committee will oversee the Operations Center on issues related to study design, progress and presentation of results, and statistical analyses. The organization of the Operations Center at Rhode Island Hospital includes the study Principal Investigator, epidemiological, administrative, and secretarial support. Dr. Bostom, as Principal Investigator and Chairman of the Executive Committee, provides medical /scientific, and epidemiological leadership. Joyce L. McKenney, MPH manages the Operations center, provides epidemiological leadership, and oversees the daily activities of the project. Persons with clinical, epidemiological, and nutritional/biochemical expertise have been recruited to be consultants to the project.
To forestall and minimize problems, one of the main responsibilities of the Operations Center is in adequate study-wide communication among clinical centers, the central laboratory, the VDC, study committees, the Data Coordinating Center, and the funding agency. This requires regular contact by e-mail, telephone, fax, mail and/or visit with all participating individuals.

The Operations Center interacts with the other investigators at semi-annual meetings. All contact with the funding institute and all administrative matters will be handled through this center. The members of the Center plus representatives from the DCC will attend the meetings of the Data and Safety Monitoring Board, *but only designated DCC representatives will participate in the confidential portion of the meeting related to interim efficacy analyses.* Site visits to the participating centers will be organized through the Operations Center. Investigators from this center will also serve on various subcommittees. The Operations Center will be linked electronically with all other centers.

We propose that this study will be a consortium between the funding agency, Rhode Island Hospital, and the collaborating organizations. The Financial Officer for the study is the Chief Financial Officer of Rhode Island Hospital who is responsible for receipt and dispersal of funds and prepares a statement of receipts and dispersals. The Fiscal Manager within the operations center is responsible for daily financial management of budgets and supervises the overall management of consortiums and total funding.

IV.A.3. Data Coordinating Center.
The Data Coordinating Center (DCC) provides epidemiological, biostatistical, data management, study management, and general scientific support for all components of the study. Technical support for the installation, use, and maintenance of local data collection equipment and software is provided by in-house staff. Dr. Lloyd Chambless heads the study's Statistical Coordinating Center, part of the Department of Biostatistics, School of Public Health, University of North Carolina at Chapel Hill.
The DCC staff participates in the activities of the Executive Committee and all subcommittees, providing technical assistance in study design, data collection, processing and analysis, training and certification, quality assurance, evaluation, and study implementation. For example, the DCC supports the End Point Committee in monitoring the status of each study end point preparing documentation of events to be verified and creating a final diagnosis file. The DCC's responsibility for the centralized management of the study includes the provision and tracking of training and certification, monitoring protocol adherence in the clinical centers, quality control in the central laboratory, and data management, including the development of a computerized data collection system, on-site and centralized data processing and data analysis. The SCC has over a 20 year record of successful collaboration with clinical centers, laboratories and other sponsoring agencies to meet the needs of the

projects for which it has been responsible. As the coordinating center for a number of multi-center medical studies, it has provided statistical, data management quality assurance, and study management services. The organization includes professional personnel from biostatistics, epidemiology, computer science/data management medicine, pharmacy, and nutrition. The professional personnel are supported by staff with training and experience in all of these fields as well as in study management office management and communications. DCC staff have been authors on over 200 peer-reviewed publications as well as several hundred presentations at scientific meetings. The DCC will perform the following functions in the support of the study:

1. Develop the study forms and the Manual of Operations with the guidance of the Steering Committee and assistance of the Operations Center.
2. Set up the distributed data entry system.
3. Train and certify clinical and laboratory personnel in the use of the data collection forms and the operation of the microcomputer system for data transmission and management support systems.
4. Receive data from all centers and the Central Laboratory and edit the data for errors.
5. Analyze the data.
6. Generate regular recruitment and quality control reports for the Steering Committee and, in addition to these, endpoint reports for the Data and Safety Monitoring Board.
7. Serve on study committees.
8. Assist in the preparation and authorship of papers, including providing statistical support.
9. Provide and facilitate communication support among the study units. In this regard, it is extremely important that the coordinators in each of the clinical centers, the Central Laboratory, and the Project Manager at the Operations Center develop a close rapport in their communications. An electronic mail media will be employed for the transmission of routine messages and for resolution of data problems. Telephone conference calls are also to be used for addressing larger problems.

IV.A.4. Central Laboratories.

The core laboratories for the study will be the adjacent Vitamin Bioavailability (VBL) and Nutrition Evaluation (NEL) Laboratories at the Jean Mayer USDA Human Nutrition Research Center on Aging (HNRCA) in Boston, Massachusetts. The principal investigator of the tHcy central laboratory (VBL) is Jacob Selhub, PhD. Gayle Perrone, MBA is a co-investigator at HNRCA who directs the Nutrition Evaluation Laboratory (NEL). Paul Jacques, ScD is a co-investigator at HNRCA who heads the Nutritional Epidemiology Program. *Random blood samples will be collected at all visits*. For screening, samples will be obtained for determination of tHcy, creatinine, and glucose, and collection of red blood cells and buffy coat specimens. For baseline and periodically throughout follow-up, samples will be collected for tHcy determinations, as well as for determination of folate, vitamin B12, and pyridoxal 5'-phosphate levels, creatinine, lipids/lipoproteins, glucose, and fructosamine. The sampling scheme for the performance of these assays from banked randomization and follow-up specimens (i.e., in the entire cohort, or within the context of specific "nested" designs) will be finalized by the Executive Committee. In addition, spot urine aliquots from baseline and follow-up visits will also be banked, and their analysis (for eg., for albumin/creatinine ratio) will be deferred pending ancillary study funding. Similarly, cells/buffy coat from the baseline and follow-up visits will also be banked for deferred analysis pending ancillary study support. Plasma (EDTA and citrate), serum, and cells/buffy coat will be aliquoted into cryopreservation tubes, frozen, and stored in the –80 degree freezers provided to each site. These specimens will be shipped to Boston in batches. While the protocol and timetable for shipment will be finalized during the 6-month protocol development and field testing phase, screening values for tHcy

and creatinine will be made available to the Data Center for transmission to the clinical centers within 2-3 weeks of specimen collection. The central laboratories will perform the following functions in support of the study: receive plasma samples, analyze (at the VBL) for tHcy, folate, B12, and PLP, as well as (at the NEL) creatinine, lipids/lipoproteins, glucose, and fructosamine, and store aliquots (both labs).

IV.A.5. Vitamin Distribution Center.
Pamlab, L.L.C. will serve as the Vitamin Distribution Center (VDC). The distribution of the vitamin supplements will be managed by the VDC. The vitamin supplements will be manufactured, bottled, labeled, and stored by Anabolic Laboratories, under the direction of Pamlab, L.L.C. The timetable for procurement and preparation of the vitamin supplies will be finalized during the protocol development phase to allow for the distribution of the prepared vitamin products in time for the planned start-up of randomization.

IV.B. Committee Organization.

IV.B.1. Executive Committee.
This is the policy and decision-making committee for the study, providing clinical and scientific direction at the operational level. Upon it sits the Principal Investigator for the Operations Center, the two key nephrologist consultants, the PIs for the Statistical Coordinating Center, and the Central Laboratory, a representative from the funding agency, and representative nephrologists/transplant surgeons from the clinical sites. This committee shares with the Principal Investigator the responsibility for overseeing performance in the study. Its membership consists of key individuals:

Andrew G. Bostom, MD, MS (Chairman) Principal Investigator	Operations Center, Rhode Island Hospital
Andrew S. Levey, MD	Nephrologist, New England Medical Center
Lawrence Hunsicker, MD	Transplant Nephrologist, University of Iowa Medical Center
Myra A. Carpenter, PhD	Epidemiologist, Data Coordinating Center UNC-Chapel Hill
Paul Jacques, ScD	Nutritional Epidemiologist, Jean Mayer USDA Human Nutrition Research Center
Marc Pfeffer, MD, PhD	Cardiologist, Director of Clinical Endpoints Core, Brigham and Women's Hospital
John W. Kusek, PhD	Project Officer, NIDDK/KUHD

The Executive Committee will meet by conference call at least bimonthly to deal with interim business (between Steering Committee meetings) to discuss the day-to-day and logistical needs of the study. Other individuals such as representatives from the Vitamin Procurement and Distribution Center, consultants, investigators from the participating centers and staff from the Operations and Statistical Coordinating Centers may be invited to attend Executive Committee meetings as ad hoc liaison.

The major responsibilities of the Executive Committee are in reviewing overall progress of the study with particular emphasis on the following activities:

(1) review and approval of any proposed revisions to the protocol or operations manual

(2) review of preliminary, non-confidential data

(3) review of reports from the Data Coordinating Center on performance of each participating institution, specifically:
   a) the rate of patient entry into the study as a whole and from each participating institution
   b) the timeliness and accuracy of data submission, including on-study data, treatment and information, follow-up reports
   c) completeness of baseline and follow-up data

   These reports will not include any data related to treatment efficacy, and will be presented in such a way as to preserve masking.

(4) identification and implementation of solutions to problems which arise, specifically:

   a) the consideration of on-site visits to those institutions with deficiencies
   b) consideration of additional institutions if one drops out or is dropped

(5) proposals to reflect additional information on patients, i.e., ancillary studies

(6) overseeing subcommittees listed below

IV.B.2. Steering Committee.
This committee consists of personnel from the Operations Center, the Data Coordinating Center, & Central Laboratories, the principal investigator from each of the participating clinical centers, as well as representatives from the patient coordinators, the funding institute, Vitamin Distribution Center, and consultants. The Steering Committee meets at least once a year throughout the study, including the period for final analysis and writing activities that follow the conclusion of patient follow-up.

To reduce costs and minimize travel time, attempts are made to schedule the meetings around professional meetings which investigators are already planning to attend, e.g., American Society of Nephrology, American Society of Transplant Physicians, etc. These meetings bring together investigators and clinical coordinators from the various participating centers for discussion regarding progress of the trial, possible changes in the protocol or methodology, new developments in the field, revitalization of interest, and other matters of concern to participants in the study. These meetings also provide an opportunity for staff training and education.

Various study design and planning committees assist the Executive Committee in such tasks as writing, revising and implementing the Manual of Operations, in standardizing diagnostic or therapeutic methodology, in monitoring the accumulation of patients, and in carrying out editorial work on abstracts, presentations and manuscripts. Much of the work of the subcommittees is handled through regular phone and e-mail/mail communications and the annual Steering Committee Meeting, but

conference calls and special meeting are called as needed. These committees, which report to the Executive Committee, are open to all investigators from the participating clinical centers and include representatives from the Operations and Data Coordinating Centers. The membership roster will be completed during the Protocol and development and field testing phase.

IV.B.3. Endpoints Verification Committee.
This committee, headed by Dr. Marc Pfeffer of Brigham & Women's Hospital Cardiovascular Division, has responsibility for the development and validation of the system for end point review and verification. Throughout the entire study, this committee advises the Executive Committee on any modifications or enhancements to the system. This committee has the responsibility for classification of whether or not a patient has reached a verified non-fatal myocardial infarction (MI) or coronary heart disease death end point, as well as for classification of whether or not a patient has reached a verified end point of non-fatal or fatal stroke. The role of this committee is ongoing throughout the entire study period. This committee analyzes the data biannually during the study to review the documentary evidence (without identity of treatment) and decision by the End Point Verification system on each patient evaluated by the system as suspect of having had an MI, coronary heart disease death, non-fatal stroke, or stroke death. Such central evaluation, with the subject's identity and intervention group assignment blinded, helps to assure unbiased classification of reported events and to eliminate problems of variable interpretation of event definition.

IV.B.4. Data and Safety Monitoring Board.
This committee will be set up by the funding institute, independent of the study investigators, to monitor the study results for evidence of adverse or beneficial treatment effects throughout the study period. The Monitoring Committee will remain "blinded" to outcome characteristics of the study for as long as possible. While the committee may have access to any information that is deemed necessary to make an appropriate determination, highly sensitive information in relation to the outcome of the study will be requested on a "need know" basis as it may arise during the course of the committee's deliberations. The committee's concerns will be directed to patient accrual, appropriate follow-up, compliance, data acquisition, undue complications, and whether the study as it is currently being conducted will be able to answer the hypothesis it addresses. The membership and frequency of meeting are at the discretion of the funding institute but will presumably consist of at least 5 members including a biostatistician, 3 clinical investigators, and a scientist from the funding institute. It is expected that this committee will meet one to two times per year and will report to the funding institute on scientific and administrative issues. For example, the Board has the responsibility for recommending early termination in case of unanticipated toxicity or greater than expected benefit. The responsibility for subject safety is particularly important, since the individual investigators are unaware of the group assignments.

IV.B.5. Additional Committees/Subcommittees.

IV.B.5.1. Recruitment Committee.
The responsibilities of this committee are to develop materials and presentations to assist investigators in recruitment of cases, and materials to encourage patient and physician interest and participation in the study. Throughout the study recruitment period, this committee advises the Executive Committee on suggested strategies to decrease deficiencies in patient accrual and where necessary, works with specific centers with particular problems.

IV.B.5.2. Quality Control Committee.
This committee will be responsible for assuring high quality data by monitoring clinic and center performance and initiating corrective action when needed. Internal quality control reports provided by the DCC will be reviewed. A system for sending blinded replicate samples to the central lab will be developed by this committee and implemented by the DCC with the results monitored by this committee.

IV.B.5.3. Publications Committee.
This committee, will formulate publication policy for this collaborative research and review all abstracts, papers and scientific presentations which utilize study data. The Publications Committee will be responsible for identifying topics for publication as well as making writing group assignments. The subcommittee will review and recommend approval or disapproval of all scientific abstracts and papers or presentations using unpublished study data, as well as every paper using published data that purports to represent official study views or policy. Another major responsibility of the Publications Committee is in the development of plans for the dissemination of trial findings and incorporation of the findings into medical care policy. This will involve not only reports in medical journals but consideration of continuing education courses, conferences and seminars and special efforts such as press conferences, editorials, physician newsletters and presentations at local medical association meetings.

IV.B.5.4. Ancillary Studies Committee.
It is anticipated that both intramural and extramural investigators will wish to capitalize on the potential for collaborative ancillary investigations afforded by the implementation of the main study. The Ancillary Studies Committee, headed by, will formally review and recommend approval or disapproval of all proposed ancillary studies, considering both their impact on the conduct of the main study, and their scientific merit.

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