Randomized Clinical Trial of Vitamin D and Omega-3 Fatty Acids for Diabetic Kidney Disease

Protocol
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Short title:
VITAL Diabetes

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Abstract

Diabetic kidney disease (DKD) is a cause of substantial morbidity and mortality. Despite widespread use of intensive glycemic control and renin-angiotensin-aldosterone system inhibitors, renal and cardiovascular consequences of DKD remain common. This study will evaluate whether vitamin D3 and/or omega-3 fatty acids are safe and effective interventions to reduce the burden of DKD among the large and growing diabetic population. Specifically, we aim to test whether vitamin D3 and/or omega-3 fatty acids prevent progression of albuminuria and loss of glomerular filtration rate, two complementary manifestations of DKD, over 4 years of treatment. To effectively and efficiently test our hypotheses, we will conduct an ancillary study to the Vitamin D and Omega-3 Trial (VITAL), an NIH-funded randomized clinical trial. In the parent VITAL study, 20,000 participants (men ages ≥ 60 years, women ages ≥ 65 years) will be randomly assigned in a 2x2 factorial design to vitamin D3 (cholecalciferol) 2000 IU daily versus placebo, and to eicosapentaenoic acid plus docosahexaenoic acid 840 mg daily versus placebo, and followed for a mean of 5 years to assess effects on cardiovascular disease and cancer events. Leveraging the established infrastructure of VITAL, the ancillary study described herein (the VITAL Diabetes sub-study) will identify and recruit a sub-cohort of 1,500 VITAL participants with diabetes at baseline. Among this VITAL subgroup, we will ascertain effects of the VITAL interventions on albuminuria and glomerular filtration rate by obtaining additional questionnaire data and urine and blood samples. Following the simple and cost-efficient design of the parent VITAL trial, biospecimens will be collected locally and delivered to the VITAL central laboratory by mail. First morning voids will be collected at baseline and year 4 for measurement of urine albumin-creatinine ratio. Blood samples will be collected simultaneously for measurement of glomerular filtration rate (using serum creatinine and cystatin C), 25-hydroxyvitamin D, eicosapentaenoic acid plus docosahexaenoic acid, C-reactive protein, and hemoglobin A1c. This ancillary study is designed to determine whether vitamin D3 and/or omega-3 fatty acids have causal and clinically relevant effects on the development and progression of DKD.

I. Background

I.a. Public health impact of diabetic kidney disease

I.a.1. *Definition of DKD.* Chronic kidney disease is defined by albuminuria or impaired glomerular filtration rate (GFR).[1] Diabetic kidney disease (DKD), defined as chronic kidney disease clinically attributable to diabetes,[1, 2] is the most common cause of kidney disease in the developed world.[3] The large majority of persons with DKD do not undergo kidney biopsy to definitively establish a pathology-based diagnosis of diabetic glomerulopathy, and some persons diagnosed with DKD have a nondiabetic cause of kidney disease. However, biopsy series confirm that the majority of persons diagnosed clinically with DKD do have diabetic glomerulopathy, and *diagnosis and treatment of DKD in practice is currently based on clinical assessment of albuminuria and GFR in the setting of diagnosed diabetes*.[1, 2, 4] The American Diabetes Association and National Kidney Foundation recommend that albuminuria be assessed as albumin-creatinine ratio (ACR) in a spot urine sample, with abnormal defined as ACR ≥ 30 mg albumin/g creatinine, and that GFR be calculated from serum creatinine, with estimated GFR < 60 mL/min/1.73m^2 defined as abnormal.[1, 2, 4]

I.a.2. *DKD is common.* The prevalence of diabetes is increasing worldwide, reaching 30% among US adults ages ≥ 60 years based on recent NHANES data (diagnosed diabetes 15-18% prevalence).[5] Individuals with diabetes are at high risk of developing DKD. In NHANES III, 42% of persons with self-reported diabetes had prevalent elevated urine ACR and/or elevated serum creatinine, compared with 14% of the unselected United States population.[6] In addition, the prevalence of kidney disease is highest and increasing most rapidly among older adults (**Figure 1**).[7] Thus, *from a public health perspective, targeting older persons with diabetes offers the highest yield opportunity to prevent and treat kidney disease and its complications*. Moreover, as the United States population continues to age, grow more obese, and develop more diabetes, the prevalence of DKD is expected to rise for the foreseeable future.[5, 7-10]

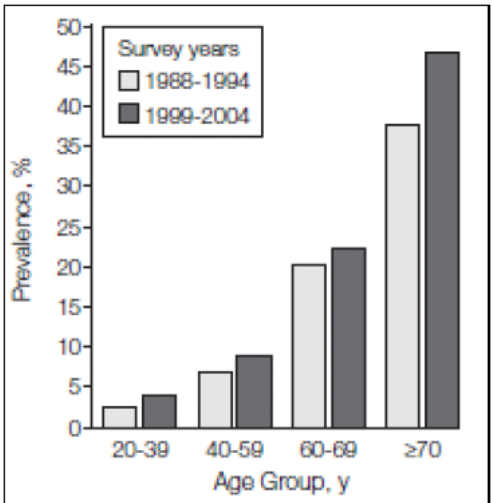

Figure 1. Prevalence of chronic kidney disease by age group in two NHANES surveys. From Coresh et al (ref 7).

I.a.3. *DKD health outcomes are poor.* The major adverse health outcomes of DKD are end-stage renal disease (ESRD), CVD, and death. The natural renal history of DKD consists of progressively increasing levels of urine albumin excretion and relentless loss of GFR.[11] While it is now clear that low levels of albuminuria ("microalbuminuria") may regress, either spontaneously or with treatment, many individuals progress to advanced stages of DKD.[3, 11-14] In 2006, DKD was the attributed cause of 49,224 cases of incident ESRD (44%) in the United States alone.[3] In addition, the presence and severity of DKD potently amplifies CVD risk.[15-18] Albuminuria and impaired GFR are each associated with increased prevalence and severity of traditional CVD risk factors, such as hypertension and dyslipidemia.[19-24] Kidney disease is also associated with novel CVD risk factors, including inflammation, oxidative stress, anemia, disordered mineral metabolism, and vascular calcification.[24-29] As a result, the prevalence and incidence of CVD are directly proportional to the severity of DKD. Moreover, as demonstrated in the United Kingdom Prospective Diabetes Study (UKPDS) and other longitudinal studies, mortality is markedly increased with greater severity of DKD (**Figure 2**).[14, 30-33] More specifically, we have demonstrated that albuminuria and estimated GFR are independently and additively associated with all-cause- and CVD-specific-mortality among older adults

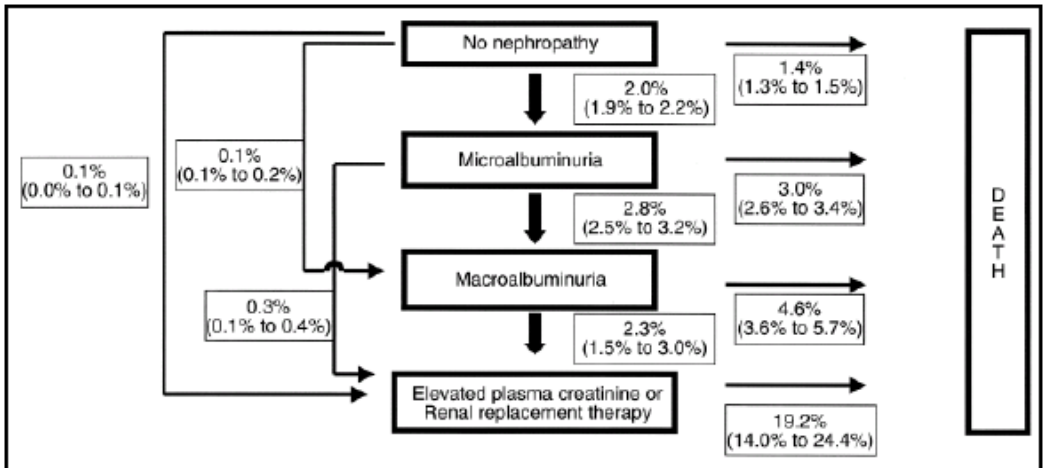

Figure 2. Mortality rates (per year) in the UKPDS, by DKD stage, from Adler et al (ref 14).

with diabetes who participated in the Cardiovascular Health Study (**Figure 3**).[34] In randomized clinical trials of renin-angiotensin-aldosterone system (RAAS) antagonists, reduction of albuminuria on therapy correlated with improved renal and CVD outcomes.[35, 36] Taken together, *these observations suggest that reducing the burden of DKD in its early stages will help prevent ESRD, CVD, and death.* Because diabetes and kidney disease are particularly strong risk factors for mortality among older adults, for whom other CVD risk factors such as hyperlipidemia have reduced prognostic utility, prevention and treatment of DKD may have particularly beneficial effects in this age group.[18, 34, 37, 38]

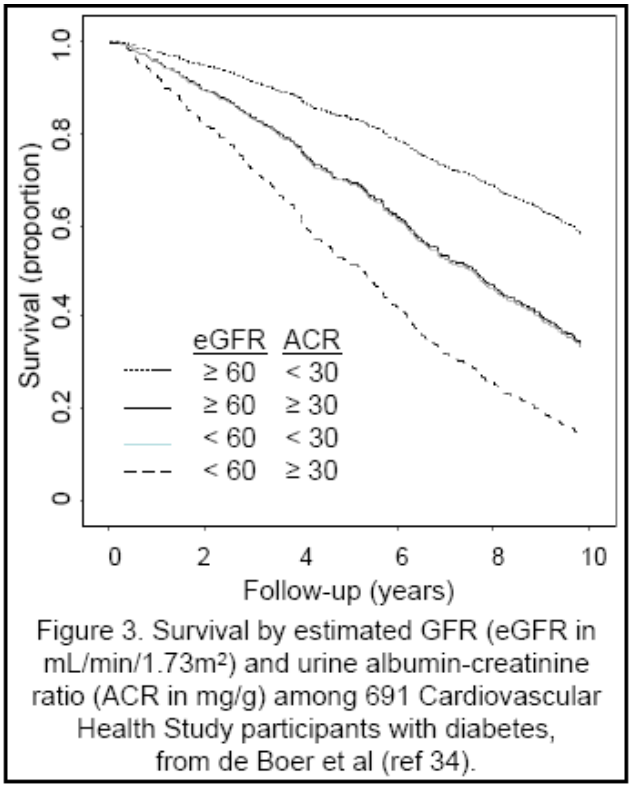

Figure 3. Survival by estimated GFR (eGFR in mL/min/1.73m²) and urine albumin-creatinine ratio (ACR in mg/g) among 691 Cardiovascular Health Study participants with diabetes, from de Boer et al (ref 34).

I.a.4. *Novel interventions for DKD prevention and treatment are urgently needed.* Current interventions to prevent and treat DKD are limited. The Diabetes Control and Complications Trial (DCCT) and the UKPDS clearly demonstrated that intensive glycemic control decreases the incidence of DKD, and perhaps its progression.[39-41] Despite these exceptionally important findings, however, the incidence of DKD in these studies remained high even in the intensive glycemic control treatment arms. For example, of DCCT participants assigned to intensive diabetes therapy, 5% had prevalent microalbuminuria (albumin excretion rate ≥ 28 µg/min) at baseline, 16% developed incident microalbuminuria during the DCCT, and an additional 7% developed incident microalbuminuria over the subsequent 8 years of follow-up.[39, 40] In addition, effects of glycemic control on prevention of CVD remain unclear: while intensive glycemic control in the DCCT resulted in a modest reduction in CVD events (myocardial infarction, stroke, CVD death, angina, coronary revascularization) over long-term follow-up, the ACCORD, ADVANCE and Veterans Affairs Diabetes Trial did not demonstrate benefits of tight glycemic control on CVD events.[41-45] In addition to glycemic control, blood pressure control is clearly important for DKD prevention and treatment. In particular, angiotensin coverting enzyme (ACE) inhibitors and angiotensin II receptor blockers are proven to prevent the development and progression of DKD.[46-51] Again, however, DKD incidence and progression remain high despite treatment with these RAAS antagonists. Therefore, *glycemic control and RAAS inhibition on their own do not meet the clinical need for DKD prevention and treatment, and novel approaches to therapy are needed. Given the high incidence of DKD among persons with diabetes, ideal new interventions would be sufficiently accessible, inexpensive, safe, and effective to apply to large numbers of persons with diabetes.*

I.b. Vitamin D is a promising therapeutic intervention for DKD prevention and treatment.

I.b.1. *Overview of vitamin D metabolism.* Vitamin D is a critical regulator of calcium, phosphorous, and bone homeostasis.[52-55] Humans derive substrate forms of vitamin D (cholecalciferol and ergocalciferol) from cutaneous synthesis and dietary intake. Cholecalciferol and ergocalciferol have little inherent biologic activity and require two hydroxylation steps for full hormonal activity. 25-hydroxylation occurs in the liver and is unregulated and non-rate-limiting. Circulating 25-hydroxyvitamin D [25(OH)D] concentration increases in proportional to UV light exposure and dietary supplement intake, has a half-life of 10-21 days, and is widely accepted as a summary measure of vitamin D intake from cutaneous and dietary sources.[56-62] Further hydroxylation to 1,25-dihydroxyvitamin D (calcitriol) occurs predominantly in the kidney and is dependent upon sufficient 25(OH)D availability, particularly among persons with kidney disease.[28, 63-68] 1,25-dihydroxyvitamin D is a pleiotropic steroid hormone that binds to the vitamin D receptor and regulates gene expression in a diverse array of target tissues.[69]

I.b.2. *Vitamin D and the kidney.* Traditional effects of 1,25-dihydroxyvitamin D include stimulation of dietary calcium and phosphorous absorption, suppression of parathyroid hormone, and maintenance of bone mineral density. Recently, the potential impact of vitamin D on metabolic and cell regulatory pathways other than those related to traditional mineral homeostasis has attracted increased attention.[55, 69-71] As one of these potential

pleiotropic actions, 1,25-dihydroxyvitamin D appears to have direct renoprotective effects – this evidence is described below and forms the rationale for Specific Aim 1. It is also possible that calcitriol deficiency due to impaired renal synthesis is a mechanism through which kidney disease leads to CVD and other adverse health outcomes – this latter hypothesis is not the focus of the current application.

I.b.3. *Mechanisms through which vitamin D may prevent DKD.* Vitamin D may prevent kidney damage by suppressing the RAAS, reducing renal inflammation, and exerting direct pro-survival effects on podocytes (**Figure 4**). First, vitamin D deficiency stimulates the transcription and release of renin in experimental animals, thus activating the RAAS, and renin is potently downregulated by administration of 1,25-dihydroxyvitamin D.[72, 73] RAAS stimulation leads to vasoconstriction, oxidative stress, and glomerulosclerosis through both hemodynamic and nonhemodynamic (e.g. fibrotic) mechanisms.[74-77] In humans, circulating 1,25-dihydroxyvitamin D concentrations are inversely correlated with blood pressure,[78-80] and lower circulating 25-hydroxyvitamin D concentrations have been associated with increased risk of incident hypertension.[81] Second, *in vitro* cell culture studies and *in vivo* animal-experimental models demonstrate potent immunomodulatory functions of vitamin D.[82] In antigen presenting cells (including monocytes), vitamin D therapy alters cytokine expression (decreased IL-1, IL-6, IL-8, IL-12, and TNF-α; increased IL-10) and regulates cell growth and cell-cell interaction (decreased differentiation, maturation, MHC-II expression, costimulatory molecule expression, and interferon-γ; increased apoptosis).[83-86] Net effects of vitamin D on immune function include inhibition of cell-mediated immunity, which contributes to the tubulointersitial fibrosis responsible for progression of DKD.[71, 87, 88] In support of this hypothesis, circulating 25(OH)D concentration is inversely correlated with renal inflammation in human kidney biopsies.[89] Third, vitamin D is known to affect cell proliferation, differentiation, and survival through regulation of cell cycle progression and modulation of apoptosis.[90-94] In animal models of kidney injury, 1,25-dihydroxyvitamin D has direct pro-survival effects on kidney podocytes, preventing damage and apoptosis.[95-100] Podocytes form a critical component of the glomerular barrier to albumin filtration, and dysfunction and loss of podocytes is a seminal feature of proteinuric kidney diseases including DKD.[101]

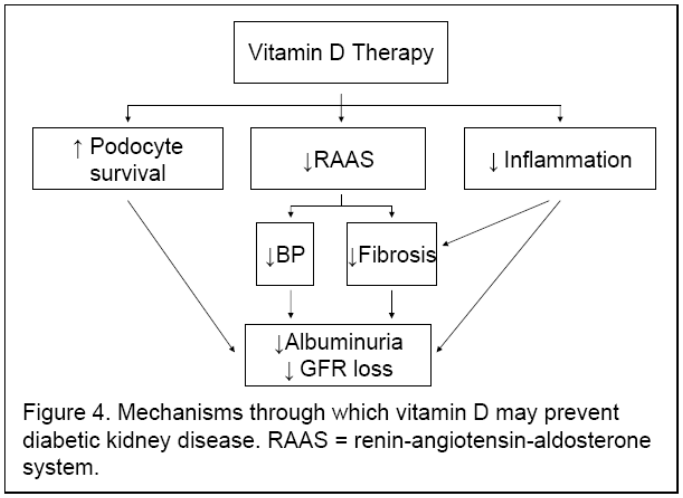

Figure 4. Mechanisms through which vitamin D may prevent diabetic kidney disease. RAAS = renin-angiotensin-aldosterone system.

I.b.4. *Vitamin D reduces albuminuria and glomerulosclerosis in animal-experimental models.* In animal-experimental models, vitamin D deficiency promotes and 1,25-dihydroxyvitamin D therapy reduces albuminuria, glomerulosclerosis, and loss of GFR.[96, 100, 102] These actions have been observed in animal models of DKD as well as the nondiabetic 5/6 nephrectomy model. Effects are mediated, at least in part, through mechanisms described in *Section B2c* and **Figure 4**. One particularly provocative study suggested that treatment with an angiotensin-II receptor blocker (losartan) and paricalcitol (an activated analogue of 1,25-dihydroxyvitamin D) acted synergistically to completely abrogate the development of albuminuria in the streptozotocin model of DKD (**Figure 5**), in part because paricalcitol prevented the compensatory increase in renal renin production normally stimulated by RAAS blockade.[102] These data raise the possibility that vitamin D interventions may prove to be particularly valuable when added to current standard DKD therapies.

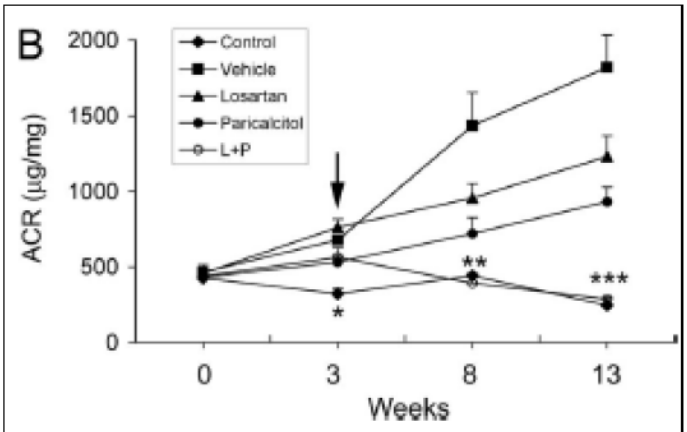

Figure 5. Losartan and paricalcitol each reduced the development of albuminuria in the streptozotocin model of DKD, while the combination (L+P) completely prevented albuminuria. From Zhang et al (ref 102).

I.b.5. *Epidemiologic studies of vitamin D and kidney disease*. Human studies examining the relationship of vitamin D with clinical manifestations of kidney disease suggest that effects observed in animal models may have clinical relevance. Comparing Italian subjects with type 1 diabetes and persistent microalbuminuria to those with normal albumin excretion rate, lower levels of both 25(OH)D (27±5 *vs* 36±5 ng/mL, p<0.01) and

1,25-dihydroxy vitamin D (25±6 *vs* 39±9 pg/mL, p<0.01) were reported.[103] Among 100 Japanese persons with type 2 diabetes, 1,25-dihydroxyvitamin D concentration was inversely correlated with urine ACR (r=-0.21, p<0.05).[104] We have demonstrated an inverse association of serum 25(OH)D concentration with albuminuria prevalence in the United States population, as represented by NHANES III (*preliminary data Section C2a*).[105] Among 168 persons with moderate-severe CKD (mean estimated GFR 34 mL/min/1.73m^2), 25(OH)D concentration was directly correlated with estimated GFR.[28]

I.b.6. *Clinical trials of vitamin D agents in kidney disease.* Two small studies reported that paricalcitol (an activated analogue of 1,25-dihydroxyvitamin D) significantly reduced albuminuria in stage 3-4 chronic kidney disease (estimated GFR 15-59 mL/min/1.73m^2). In the first study, among 118 participants with dipstick positive albuminuria at baseline, urine dipstick results improved among 51% of participants assigned to paricalcitol versus 25% of participants assigned to placebo after 24 weeks of therapy (p=0.004).[106] In the second study, 24-hour urine albumin excretion rate increase by 35% over 1 month among participants assigned to placebo and decreased by 48% and 46% among participants assigned to paricalcitol 1 mcg and 2 mcg daily, respectively (8 participants per group, p<0.001).[107] An industry-sponsored clinical trial is currently recruited to follow up on these data by testing whether paricalcitol reduces albuminuria among participants with advanced DKD (diabetes with urine ACR 100-3000 mg/g and estimated GFR 15-90 mL/min/1.73m^2) over 1 year.[108] While results of this trial may have important implications for the studies proposed in this application, they will not determine whether cholecalciferol prevents the development or progression of DKD among persons with early or inapparent DKD, for whom renal activation of 25(OH)D to 1,25-dihydroxyvitamin D is largely preserved (thus abrogating the need for an activated form of vitamin D) and for whom activated vitamin D analogues may be associated with unnecessary risk and cost. Currently, there are no published clinical trials data examining effects of cholecalciferol or ergocalciferol treatment on the presence or severity of diabetic or other forms of kidney disease.

I.b.7. *Race/ethnicity, vitamin D, and DKD.* 25-hydroxyvitamin D concentration varies strongly by race/ethnicity due to differences in skin pigmentation and cutaneous cholecalciferol synthesis, being highest in Caucasian populations, intermediate in Hispanic and Asian populations, and lowest in African American populations.[68, 105, 109] The prevalence and incidence of chronic kidney disease in general and DKD specifically also vary strongly by race/ethnicity, with higher rates among minority populations.[3, 110-113] Given these observations, and the biologic hypothesis that vitamin D helps prevent DKD, it is possible to speculate that vitamin D deficiency mediates, in part, the increased prevalence and incidence of DKD in non-Caucasian populations. If so, vitamin D supplementation may reduce this racial and ethnic disparity.

I.c. Omega-3 fatty acids are a promising therapeutic intervention for DKD prevention and treatment.

I.c.1. *Omega-3 fatty acids (ω-3 FA).* Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 long chain polyunsaturated fatty acids which are found mainly in fatty fish. Diets rich in ω-3 FA or supplemented with ω-3 FA have been associated with diverse health benefits, including prevention of type 2 diabetes, CVD, dyslipidemia, and thrombosis.[114-116] Recently, it was reported that EPA 1.8 grams daily reduced the incidence of major CVD events by 19% among persons with elevated LDL cholesterol, compared with placebo (JELIS trial).[117]

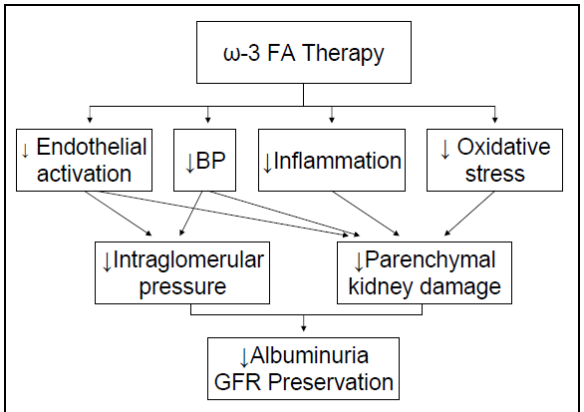

Figure 6. Mechanisms through which omega-3 fatty acids (ω-3 FA) may prevent diabetic kidney disease.

I.c.2. *Potential reno-protective actions of ω-3 FA.* Intervention studies in humans suggest that ω-3 FA have a number of actions that may directly help prevent the development and progression of kidney disease. These include suppression of endothelial activation, reduction of renal inflammation and oxidative stress, and modest blood pressure lowering (**Figure 6**). First, at least 7 human intervention studies suggest that ω-3 FA improve vasodilation and/or decrease markers of endothelial cell activation.[114] For example, in a cross-over trial among 23 participants with type 2 diabetes, six weeks of ω-3 FA administration improved forearm blood flow responses to acetylcholine, when compared to the vasodilator responses recorded at baseline or after olive oil administration.[118] Another study reported that ω-3 FA significantly reduced soluble cell adhesion molecules, particularly among persons with type 2 diabetes.[119] Endothelial

function regulates intraglomerular pressure, and the endothelium is a critical barrier to urinary albumin filtration. Second, at least 3 studies of persons with type 2 diabetes found that ω-3 FA reduced measures of oxidative stress, such as urine F2-isoprostane excretion.[120-122] Oxidative stress is closely linked with tissue inflammation (*Section B2c*) and may contribute to the pathogenesis of albuminuria and DKD progression.[25, 88] Third, ω-3 FA appear to have modest effects lowering systemic blood pressure, perhaps related to the mechanisms described above.[114]

I.c.3. *ω-3 FA reduce albuminuria and tubulo-interstitial fibrosis in animal-experimental models.* Effects of ω-3 FA have recently been studied in several animal models of kidney disease. In the diabetic KKay/Ta mouse, intraperitoneal EPA reduced mesangial expansion, albuminuria, and tubulo-interstitial fibrosis.[123] It was postulated that these effects were mediated by suppression of monocyte chemoattractant protein-1 (MCP-1), a protein which regulates macrophage recruitment, is increased in experimental DKD, and was suppressed by EPA administration. In the stretozotocin model of DKD, ω-3 FA prevented the development of albuminuria (urine albumin excretion approximately 10 mg/d, versus 95 mg/day in untreated and 17 mg/day in non-diabetic control animals), reduced glomerulsclerosis, and downregulated MCP-1, interleukin-6, transforming growth factor-β (TGF- β), and collagen type I and type IV.[124] In the 5/6 nephrectomy model of nondiabetic kidney disease, ω-3 FA suppressed markers of renal inflammation, including MCP-1; downregulated tissue-level NAD(P)H oxidase; and reduced expression of fibrotic markers including TGF-β.[125] Together, these studies suggest that ω-3 FA reduce kidney disease in animals by reducing renal oxidative stress and inflammation.

I.c.4. *Epidemiologic studies of ω-3 FA and kidney disease.* Observational data suggest that effects of ω-3 FA on the diabetic kidney may have clinical relevance. A cross-sectional study of 22,384 individuals in the European Prospective Investigation of Cancer-Norfolk population cohort demonstrated that greater dietary fish intake was associated with decreased risk of macroalbuminuria (urine ACR ≥ 220 mg/g) among participants with diabetes.[126] In the InCHIANTI study, a population-based Tuscan cohort, higher baseline plasma total polyunsaturated fatty acids, ω-3 FA, and ω-6 FA were each strong independent predictors of slower loss of creatinine clearance over 3 years of follow-up (P <0.0001).[127]

I.c.5. *Clinical trials of ω-3 FA for the treatment of kidney disease.* ω-3 FA have been studied in small clinical trials to treat established DKD, IgA nephropathy, and other glomerular diseases. Individually, these trials have not generated consistent findings. However, a recent meta-analysis of 17 clinical trials (626 total participants) reported that ω-3 FA significantly reduced albuminuria.[128] Effects on albuminuria in 7 clinical trials of persons with diabetes (222 participants) were consistent with the overall estimate of ω-3 FA effect (**Figure 7**).[129-135] 12 of 17 clinical trials additionally reported effects on GFR; in these, ω-3 FA resulted in a preservation of GFR, relative to control groups, which was not statistically significant. Studies included in the meta-analysis were heterogenous with regard to population, precise nature and dose of intervention, follow-up, and outcome ascertainment, such that results require confirmation in adequately powered, high-quality clinical trials. No definitive large clinical trials of ω-3 FA in diabetic or other forms of kidney disease have been published.

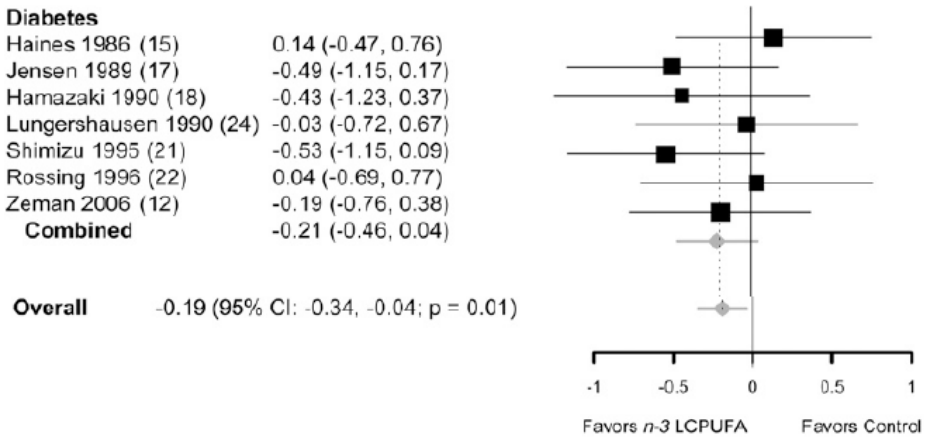

Figure 7. Meta-analysis of clinical trials assessing effects of omega-3 fatty acids on albuminuria in Diabetes ("Combined") and all glomerular disease ("Overall"), from Miller et al (ref 128).

I.d. Summary of background and current gaps in knowledge. DKD is highly prevalent, particularly among older adults, and leads to substantial morbidity and mortality due to progressive kidney and cardiovascular disease. While tight glycemic control and RAAS inhibitors help prevent the incidence and progression of DKD, rates of adverse renal and cardiovascular consequences remain unacceptably high. Cholecalciferol and ω-3 FA are promising interventions for the prevention and treatment of DKD, because

animal-experimental data suggest they have substantial reno-protective effects, early human data suggest these effects have clinical relevance, and each intervention is relatively accessible, inexpensive, and safe. Thus, it is possible that cholecalciferol and/or ω-3 FA are appropriate for wide-spread use to prevent and treat DKD in the large and growing population of older persons with diabetes. This hypothesis requires rigorous testing in well-controlled clinical trials.

II. SPECIFIC AIMS

Diabetic kidney disease (DKD) is a common and morbid complication of diabetes mellitus. Preventing the development and progression of DKD is a critical goal for improving long-term health outcomes of the growing diabetes population and for reducing the overall burden of kidney disease in the United States. Therefore, there is an urgent need for additional, complementary interventions to prevent and treat DKD. This need is greatest among older adults because diabetes and kidney disease are highly prevalent among older adults and kidney disease is among the strongest risk factors for mortality in this group. Vitamin D and omega-3 fatty acids (ω-3 FA) are two of the most promising interventions for DKD prevention and treatment. Because cholecalciferol and ω-3 FA are relatively safe, inexpensive, and widely available, they may offer opportunity to substantially reduce the burden of DKD in large populations. Prior to widespread implementation, however, these hypothesized benefits should be rigorously tested in well-powered clinical trials. Therefore, among older adults with diabetes, we aim:

SPECIFIC AIM 1: To test whether cholecalciferol (vitamin D_3, 2000 IU per day) prevents the development and progression of DKD, compared with placebo.

Hypothesis 1a: Cholecalciferol slows progression of albuminuria over 4 years of therapy.

Hypothesis 1b: Cholecalciferol reduces loss of GFR over 4 years of therapy.

Hypothesis 1c: Beneficial effects of cholecalciferol are strongest among persons who at baseline have low plasma 25-hydroxyvitamin D concentration, albuminuria, and/or impaired GFR.

SPECIFIC AIM 2: To test whether marine omega-3 fatty acids (ω-3 FA, eicosapentaenoic acid plus docosahexaenoic acid, 840 mg per day) prevent the development and progression of DKD, compared with placebo.

Hypothesis 2a: ω-3 FA acids slow progression of albuminuria over 4 years of therapy.

Hypothesis 2b: ω-3 FA reduce loss of GFR over 4 years of therapy.

Hypothesis 2c: Beneficial effects of ω-3 FA are strongest among persons with low baseline levels of plasma eicosapentaenoic acid and docosahexaenoic acid and/or high baseline levels of C-reactive protein.

To test these hypotheses effectively and efficiently, we propose an ancillary study to the Vitamin D and Omega-3 Trial (VITAL). The parent VITAL trial will be a randomized, double-blind, placebo-controlled trial of the benefits and risks of cholecalciferol and ω-3 FA in the primary prevention of cardiovascular disease and cancer. Funded by the NIH (R01 CA138962), 20,000 participants (men ages ≥ 60 years, women ages ≥ 65 years) will be randomly assigned in a 2x2 factorial design to cholecalciferol 2000 IU daily versus placebo, and to eicosapentaenoic acid plus docosahexaenoic acid 840 mg daily versus placebo. Planned mean follow-up is 5 years. Leveraging the established infrastructure of VITAL, we propose to ascertain effects of cholecalciferol and ω-3 FA on albuminuria and GFR among 1,500 participants with diabetes at baseline. Following the simple and cost-efficient design of the parent VITAL trial, biospecimens will be collected locally and delivered to the VITAL central laboratory by mail. First morning voids will be collected at baseline and year 4 for measurement of urine albumin-creatinine ratio. Blood samples will be collected simultaneously for measurement of GFR (using plasma creatinine and cystatin C), 25-hydroxyvitamin D, eicosapentaenoic acid plus docosahexaenoic acid, C-reactive protein, and hemoglobin A1c.

This VITAL Diabetes ancillary study is designed to determine whether cholecalciferol and ω-3 FA have causal and clinically relevant effects on the development of DKD. Null results of the studies proposed herein would suggest that salutary effects of vitamin D and/or ω-3 FA observed in animal-experimental studies do not translate to meaningful clinical benefits in early DKD. Positive results would support wide-spread use of cholecalciferol and/or ω-3 FA interventions among the growing and high-risk population of persons with diabetes.

III. Study Population

III.a. *Parent VITAL study population.* The study described herein is an ancillary study to the NIH-funded VITAL study. Therefore, all participants in our ancillary study must be existing participants in the parent VITAL study. The parent VITAL study will be conducted among 20,000 apparently healthy participants. The study population is restricted to older individuals (men ages ≥ 60 years, women ages ≥ 65 years), because rates of chronic disease (including CVD, cancer, and kidney disease) increase substantially with age. VITAL will exclude persons with clinically apparent CVD or cancer (except non-melanoma skin cancer), because it is a trial for the primary prevention of these conditions. Other eligibility criteria, which focus largely on safety, are listed in **Table 1**. VITAL will oversample for participants of black and Hispanic race/ethnicity.

Table 1. Eligibility criteria

Inclusion criteria
* Age ≥ 60 years (men) or ≥ 65 years (women)
* High school education or higher (for questionnaires)
* Willing to limit nonstudy vitamin D intake to ≤ 800 IU/d
* Willing to limit calcium intake to ≤ 1200 mg/d
* Willing to forego fish oil supplements
* Adherence to placebo run-in study medication > 67%

Self-reported history of diabetes
Blood and urine specimens returned during placebo run-in

Exclusion criteria
* Known CVD: MI, stroke, TIA, angina pectoris, CABG, PCI
* History of cancer, except non-melanoma skin cancer
* History of kidney stones
* Kidney failure or dialysis
* Hypercalcemia
* Hypo- or hyperparathyroidism
* Cirrhosis of the liver
* Granulomatous diseases (sarcoidosis, tuberculosis)
* Fish allergy (for EPA + DHA)
* Other serious illnesses which preclude participation

Diagnosis of diabetes only during pregnancy
Diabetes diagnosis prior to age 30 and first treated with insulin
Known cause of kidney disease other than diabetes
Kidney transplant

* Parent VITAL study eligibility criteria

The VITAL study population will be recruited entirely by mail. A master mailing tape containing 1.2 million names and addresses will be assembled by Listmart, a list broker with access to all commercially available U.S. mailing lists. This master mailing tape will be compiled from the same age-selected source tapes as the VITAL pilot study (i.e., licensed health professionals, other professionals, AARP members, and *Essence* subscribers), as well as subscription lists of magazines that appeal to professionals and college-educated individuals (e.g., *National Geographic* and *Time*) and a subset of Listmart's executive database that includes black business professionals. Each of the 1.2 million persons on the master mailing tape will be mailed a letter explaining the rationale for VITAL, an informed consent form, a brief questionnaire, and a self-addressed, pre-paid envelope for returning study forms. The letter will outline what participation would entail and provide sources for further information on relevant scientific issues. Persons identified as black with a high degree of certainty (e.g., *Essence* subscribers) will receive a special invitation letter emphasizing that blacks are at higher risk for vitamin D deficiency, as well as heart disease, stroke, and certain cancers. The initial questionnaire will focus only on VITAL eligibility criteria, containing items related to demographics, specific medical history (including diabetes), relevant allergies, and dietary intakes of vitamin D and fish, as well as contact information. Based on the completed pilot mailing, it is anticipated that roughly 40,000 individuals (25% minority race/ethnicity) will be willing and eligible for the placebo run-in.

III.b. *VITAL Diabetes ancillary study population.* Eligibility criteria specific to this proposed ancillary study are listed along with parent VITAL trial eligibility criteria in **Table 1**. We aim to recruit a diabetes sub-cohort with prevalent diagnosed type 2 diabetes. Persons with known diabetes are at high risk of DKD and comprise a targetable population for clinical intervention. For the purpose of this VITAL Diabetes ancillary study, we will define diabetes as a self-reported physician diagnosis of diabetes. Specificities of self-reported diabetes diagnosis were 97.5% and 98.9% in the WHS and NHS, respectively, using self-report alone.[136] We will exclude (a) persons who report a diagnosis of diabetes only during pregnancy (presumed gestational diabetes), and (b) persons who report diabetes diagnosis prior to age 30 and first treated with insulin (likely type 1 diabetes). Persons who are likely to have type 1 diabetes will be excluded because DKD in this population generally develops prior to older age among susceptible individuals, thus leading to disease heterogeneity compared with the much larger proportion of participants with type 2 diabetes. To focus on DKD, we will also exclude (a) participants who report a physician diagnosis of kidney disease caused by a

condition other than diabetes, and (b) participants who have had a kidney transplant.

III.c. *Recruitment of the VITAL Diabetes ancillary study.* To conduct the VITAL Diabetes ancillary study outlined in this application, we will identify, recruit, and study a sub-cohort of 1,500 VITAL participants with pre-existing diabetes at baseline. The VITAL Diabetes ancillary study sub-cohort will be recruited during the 3- to 6-month placebo run-in phase of the parent VITAL trial. VITAL will utilize a 3-6 month placebo run-in phase to maximize the proportion of randomized participants who adhere to study medications. Maximizing adherence increases the power of the study, because study outcomes will be analyzed according to treatment assignment (intention to treat).[137] During the run-in phase, all participants will be asked to take placebo cholecalciferol and placebo ω-3 FA. Participants will receive a follow-up questionnaire reevaluating eligibility criteria and assessing willingness to continue in the trial, pill adherence, and potential adverse effects. Parent VITAL investigators estimate that, of the 40,000 initially willing and eligible individuals enrolled in the run-in, 50% (n=20,000) will be adherent and remain willing and eligible for randomization. The placebo run-in phase provides a critical window of opportunity for ancillary study recruitment.

Persons willing to participate in the parent VITAL trial will return a screening medical history form concurrent with their parent VITAL trial consent form. Those who respond “YES” to the question “Have you EVER been diagnosed as having diabetes?” and who indicate "YES" or "MAYBE" to the question "Are you willing to give a blood sample" (in addition to meeting initial parent VITAL trial eligibility criteria) will be targeted for VITAL Diabetes ancillary study recruitment. With their placebo run-in materials, we will send these persons a DKD ancillary study packet, which includes:

- Letter explaining the rationale for the VITAL DKD ancillary study,
- DKD ancillary study informed consent form,
- DKD ancillary study questionnaire,
- Urine collection kit, and
- Self-addressed, pre-paid FedEx box for returning study materials.

The VITAL Diabetes ancillary study letter will outline what participation in the VITAL Diabetes ancillary study would entail and will provide sources for further information on relevant scientific issues. It will explain that participation in the ancillary study is optional and that it will not affect participation in the parent VITAL trial. The VITAL Diabetes ancillary study questionnaire will include items which clarify the self-reported diagnosis of diabetes and further define diabetes and kidney disease history. All persons will have the option to decline VITAL Diabetes ancillary study participation by checking a single box on their initial contact letter and returning this by mail to the VITAL coordinating center, or by calling the toll-free VITAL telephone number. Persons who do not respond to the first mailing of VITAL Diabetes ancillary study materials will be sent a reminder mailing and/or contacted by telephone.

Recent NHANES estimates suggest that 15-18% of the US population in the VITAL study age range have been diagnosed with diabetes, not including 13% with undiagnosed diabetes.[5] Based on VITAL recruiting strategies and selective response to mailings, it is possible that the VITAL population will be somewhat healthier than the general US population. This may have competing effects on the number of VITAL participants with diagnosed diabetes, with a lower prevalence of diabetes overall but a higher proportion of diabetes being diagnosed. Accordingly, of the 20,000 participants who will be enrolled in VITAL, we conservatively estimate that 3,000 participants will have prevalent diagnosed diabetes. VITAL Diabetes ancillary study participant burden is modest compared with parent VITAL trial activities (including study interventions), and VITAL participants with diabetes may be further motivated to contribute to scientific advancement in a disease with which they have personal experience. Thus, we anticipate that the majority of VITAL participants with diabetes will consent to VITAL Diabetes ancillary study participation, allowing quick and successful recruitment of the VITAL Diabetes ancillary study population (n=1,500).

IV. Subject enrollment

IV.a. *Methods of Enrollment.* All potential participants who return a valid VITAL Diabetes ancillary study consent form will be evaluated for enrollment in the VITAL Diabetes ancillary study. First, in collaboration with study staff for the parent VITAL study, we will assess whether each potential participant qualifies for the parent trial. Second, we will screen responses to the initial diabetes questionnaire using computer algorithms, excluding potential participants who report a diagnosis of diabetes only during pregnancy, diabetes diagnosis prior to age 30 and first treated with insulin, a known cause of kidney disease other than diabetes of hypertension, or kidney transplantation. Follow-up telephone calls will be used to clarify incomplete or inconsistent responses. Finally, we will include only participants who during the placebo run-in period return samples of both blood (for the parent VITAL trial) and urine (for this VITAL Diabetes ancillary study). Follow-up telephone calls will be used to remind potential participants to complete blood and urine collections, if needed. The first 1,500 VITAL participants who meet these VITAL Diabetes ancillary study eligibility criteria (**Table 1**) will be fully enrolled in the VITAL Diabetes ancillary study. Persons who meet eligibility criteria only for the parent VITAL trial will continue in the parent trial only.

IV.b. *Informed Consent.* A VITAL Diabetes consent form will be mailed to each potential participant as part of their initial VITAL Diabetes ancillary study packet. All persons will have the option to decline VITAL Diabetes ancillary study participation by checking a single box on their initial contact letter and returning this by mail to the VITAL coordinating center, or by calling the toll-free VITAL telephone number. The initial contact letter and consent form will encourage participants to ask questions or address concerns by calling the toll-free VITAL telephone number to speak with study staff in person. Willing potential participants will return their consent forms to the VITAL coordinating center by mail. Study staff will contact potential participants by telephone to clarify ambiguous or invalid consent forms.

IV.c. *Treatment Assignment.* Upon enrollment in the VITAL Diabetes ancillary study and/or parent VITAL trial, participants will be randomly assigned to vitamin D and omega-3 fatty acid treatment groups by the parent VITAL trial. Assignment to treatment groups will be accomplished using a computer-generated table of random numbers, stratified by 5-year age group. Within each age group, treatment assignments will be generated in blocks of eight individuals, with two individuals in each of the four treatment combinations: cholecalciferol + ω-3 FA, cholecalciferol + placebo, placebo + ω-3 FA, or placebo + placebo.

V. Study Procedures

V.a. *Approach to Data Collection.* We will to collect all study data via mail, including questionnaires and urine and blood samples. The BWH Division of Preventive Medicine research group has extensive experience collecting and analyzing questionnaires and collecting, processing, and storing biospecimens using the mail-based collection procedures to be used in this study. We will utilize these established, standardized protocols, described below, for all data collection.

V.b. *Questionnaire Data.* All participants will be asked to complete diabetes questionnaires at baseline and after 4 years of study participation. Questionnaires will address diabetes diagnosis, diabetes history, history of kidney disease, and medication use related to diabetes and kidney disease.

V.c. *Urine Collection.* Each participant will be sent a standardized urine collection kit at baseline (pre-randomization) and again after 4 years of study participation. The kit will include instructions for proper collection of a clean-catch first morning void; a urine cup; a plastic single-use pipette; two 5-mL screw-top cryovials; a gel-filled freezer pack; and an overnight courier air bill. Participants will be instructed to fill each 5 mL cryovial with urine using the pipette, to securely close each cryovial, and to immediately mail both vials to the VITAL laboratory at the BWH Division of Preventive Medicine in the freezer packs provided. Upon receipt, any precipitate in the urine will be removed by centrifugation, and the urine will be aliquoted without preservative into six 2-ml Nunc vials. Urine samples will be thawed for measurements of albumin and creatinine.

V.d. *Blood Collection.* Each participant will be sent a standardized blood collection kit after 4 years of study participation. This kit will be identical to the blood collection kit used by the parent VITAL trial for collection of baseline (pre-randomization) blood samples. (Baseline blood samples will be collected only by the parent VITAL trial under the parent VITAL trial IRB approval and with separate informed consent. These samples will be made available for use in the VITAL Diabetes ancillary study.) The kit will include supplies and instructions for having blood drawn into two EDTA tubes, one heparin tube, and one citrate tube; a gel-filled freezer pack; and an overnight courier air bill. We anticipate that most participants will have their blood drawn by their own healthcare providers or in another healthcare or blood-drawing facility. Participants will be instructed to record the time of venipuncture and the time of their last meal. They will be instructed to immediately send their sample to the VITAL laboratory at the BWH Division of Preventive Medicine in the freezer packs. Upon receipt, samples will be processed using the same protocols as the parent VITAL trial, in order to avoid laboratory errors. Blood samples will be centrifuged to separate plasma and red blood cells, each aliquoted into 2-ml Nunc vials. EDTA-plasma samples will be thawed for measurements of creatinine, cystatin C, C-reactive protein, 25-hydroxyvitamin D, and eicosapentaenoic acid and docosahexaenoic acid. Red blood cells will be thawed for measurement of hemoglobin A1c.

V.e. *Interventions.* All interventions will be provided by the parent VITAL trial and are included in the IRB application for the parent VITAL trial. For completeness, these are described below.

V.e.1. *Cholecalciferol intervention*. Active cholecalciferol (vitamin D_3) and matching inert placebo will be provided by Pharmavite LLC. Cholecalciferol is recommended by many experts as the optimal form of vitamin D supplementation based on its known dose-response effects on 25(OH)D concentration, documented long-term safety, and proven benefits in fracture reduction when used with calcium in the setting of osteoporosis.[61, 62, 138] Each active cholecalciferol study pill will contain 2000 IU (40 µg) cholecalciferol. This dose was chosen to maximize the separation in 25-hydroxyvitamin D concentration attained between treatment groups while maintaining safety. Specifically, cholecalciferol 2000 IU daily is conservatively anticipated to raise mean 25-hydroxyvitamin D concentration by approximately 16 ng/mL, while maintaining total daily cholecalciferol intake at or below the no-observed-adverse-effect level (NOAEL) specified by the Institute of Medicine, and well below the 4000 IU NOAEL specified by the European Commission Scientific Committee on Food, with allowance for nonstudy use up to 800 IU/d.[62, 138-140] Study medications will be dispensed in calendar packs, with participants asked to take one pill (cholecalciferol or placebo) each day. Participants will be instructed to discontinue their study pills if, during follow-up, they receive a diagnosis of kidney stones or hypercalcemia.

V.e.2. *Omega-3 fatty acid intervention.* Active fish oil (1000 mg capsule containing 840 mg EPA + DHA) and matching inert placebo will be provided by Pronova BioPharma ASA of Norway. Each active soft-gel capsule will contain a total EPA+DHA dose of 840 mg/d, the highest dose of EPA + DHA currently available in a 1000 mg capsule. We believe that this dose provides the best balance of feasibility, efficacy and safety. We seek to

obtain a large-enough difference in omega-3 fatty acid status between the treatment and placebo groups to detect health benefits, and there appear to be few safety issues associated with this dose. Health authorities recommend 400 mg to 1 g/d for cardioprotection.[141] A total dose of 840 mg/d was used in the GISSI-Prevenzione Trial (EPA to DHA ratio, 1:2) and AREDS 2 (EPA to DHA ratio, 2:1), while a dose of 1.8 g/d of EPA was used in JELIS. For this trial, we have selected a total dose of omega-3 fatty acids recommended by the American Heart Association for cardioprotection and demonstrated to be beneficial in one secondary prevention population with minimal side effects.[142] Because the optimal ratio of EPA to DHA is unknown, we have selected a 40:30 ratio of EPA to DHA. The ratio of omega-3 to omega-6 fatty acid intake is between 1:10 and 1:20 in most Western countries, including the U.S., whereas the optimal ratio for disease prevention has been hypothesized to be closer to 1:1 or 1:2, although this is controversial. Indeed, there is growing consensus that the absolute intake of omega-3 is a more important predictor of health than is the ratio of omega-3 to omega-6 intake, at least for cardiovascular outcomes. However, given that the average intake of EPA+DHA is 100-200 mg/d among U.S. adults, the proposed intervention of 840 mg/d would be expected to increase the average participant's omega-3 intake by a factor of 4 to 8. Assuming no concurrent change in omega-6 intake, the proposed omega-3 dose would thus have the effect of achieving the purported optimal omega-3 to omega-6 ratio and providing intakes associated with benefits in previous studies.

As with vitamin D, potential side effects of omega-3 fatty acids are rare. They include gastrointestinal (GI) symptoms (stomach upset or pain, nausea, constipation, diarrhea), bleeding (any GI bleed, GI bleeding requiring transfusion, hematuria, easy bruising, epistaxis), skin rash, colds or upper respiratory tract infection, flu-like symptoms, bad taste in mouth, and physician diagnosis of atrial fibrillation or other irregular rhythms. The FDA has concluded that marine omega-3 fatty acid doses of up to 3 g/d are "Generally Recognized as Safe." The AHA also has concluded that these risks are very low or low at doses of up to 1 g/d and low to moderate at doses of 1-3 g/d. Because they undergo an extensive purification process, high-quality fish oil supplements are free of environmental toxins (e.g., methylmercury, polychlorinated biphenyls [PCBs], and dioxins) found in some fish. The ω-3 FA supplement contains no vitamin D.

VI. Analysis plan

VI.a. *Descriptive analyses.* We will describe creation of the VITAL diabetes sub-cohort according to CONSORT guidelines, including numbers of participants who consent to VITAL participation; self-identify a diagnosis of diabetes; consent to participation in the DKD ancillary study; complete baseline urine and blood collections; and complete year 4 urine and/or blood collections. We will document reasons that participants do not complete the proposed studies, e.g. death, withdrawal of consent, or loss to follow-up. Among participants recruited into the DKD ancillary study, we will compare baseline characteristics of those who do and do not complete year 4 biospecimen collections in order to assess the potential impact of loss to follow-up. Among participants who complete year 4 biospecimens, we will describe by treatment assignment baseline characteristics and changes in clinical characteristics over the course of the study.

VI.b. *Primary study outcome.* The primary outcome of this study is change in urine albumin-creatinine ratio (ACR) from baseline to year 4, analyzed as a continuous variable. We will examine percent change in urine ACR, because relative increases in urine ACR have been roughly linearly associated with adverse health parameters and outcomes in the published literature, and because the distribution of urine ACR and its change over time are normalized by log transformation.[21, 38, 143, 144] Percent change in urine ACR will be calculated and summarized by treatment group as $[(ACR_4 - ACR_0) / ACR_0]$, where ACR_4 represents urine ACR at follow-up (year 4) and ACR_0 represents urine ACR at baseline.

VI.c. *Secondary study outcomes.* As a secondary dichotomous outcome, we will examine change in estimated glomerular filtration rate (eGFR) from baseline to year 4, analyzed as a continuous variable. We will measure plasma concentrations of both creatinine and cystatin C to estimate GFR using the most precise serologic methods available. Specifically, data from multiple community-based studies have been combined to create and validate an equation which calculates estimated GFR from serum creatinine and cystatin C, along with demographic variables.[145]

Equation 1: Estimated GFR = $177.6 \times SCr^{-0.65} \times CysC^{-0.57} \times age^{-0.20} \times (0.82 \text{ if female}) \times (1.11 \text{ if black})$

where SCr is serum creatinine (mg/dL) and CysC is cystatin C (mg/L). GFR estimated using this equation captures 89% of variation in GFR measured using radionucleotide methods. In addition, use of serum creatinine and serum cystatin C in combination to estimate GFR offers important theoretical advantages. Serum cystatin C concentration appears to be more sensitive to mild decrements in kidney function than serum creatinine, such that inclusion of cystatin C may aid detection of changes in GFR within the "normal" range.[146-148] However, some reports suggest that serum cystatin C concentration may be affected by systemic inflammation.[149, 150] This influence may be moderated by combination with creatinine. We will examine change in eGFR without transformation, because absolute differences in eGFR have been roughly linearly associated with adverse health parameters and outcomes in the published literature, and because the distribution of eGFR and its change over time is roughly normal.[18, 34, 37] Change in eGFR will be calculated and summarized by treatment group as $[eGFR_4 - eGFR_0]$.

As an additional secondary outcomes, we will examine the proportion of participants who develop a composite outcome of albuminuria progression (100% increase in urine ACR *and* a year 4 urine ACR ≥ 30 mg/g), rapid loss of eGFR (loss of eGFR ≥ 12 mL/min over the 4-year interval), ESRD (maintenance dialysis or kidney transplant verified by medical records review), and/or death. This composite outcome serves two purposes. First, it addresses large changes in DKD which have particularly high clinical impact. Second, the composite outcome reduces the potential impact of competing risks that preclude assessment of urine ACR and/or eGFR at follow-up. Accounting for competing risks will be particularly important if the incidence of ESRD and/or death differs by treatment assignment. The composite endpoint is scientifically important, because each component is a clinically relevant diabetes outcome and progression of DKD is strongly associated with ESRD and death. A 100% increase in urine ACR is generally considered to have clinical relevance, and additional inclusion of a threshold (30 mg/g) will reduce the potential impact of measurement error among persons with "normal" levels of albuminuria near the limits of assay reliability. Loss of eGFR exceeding 12 mL/min over the 4-year interval (3mL/min/year) has been associated with increased risk of mortality among older adults with and without diabetes.[34, 37]

VI.d. *Interaction of cholecalciferol and ω-3 FA.* We will assess for interaction of the study interventions (vitamin D and ω-3 FA) for each of the primary and secondary study outcomes. First, we will assess for interaction on the additive scale. Differences in each study outcome comparing the treatment group which

receives both active study medications (vitamin D + ω-3 FA) to the treatment group which receives neither (placebo + placebo) will be compared to the sum of differences comparing treatment groups receiving only one medication (vitamin D + placebo, or placebo + ω-3 FA) to the treatment group which receives neither (placebo + placebo). Interaction on the additive scale exists if the former difference differs significantly from the sum of the latter two differences.[151] Second, we will assess for interaction on the multiplicative scale by testing the joint effect of assigned treatment on each study outcome using ANCOVA. For urine ACR:

Equation 2: $log(ACR_4) = \beta_0 + \beta_1 log(ACR_0) + \beta_2(vitamin\ D) + \beta_3(\omega\text{-}3\ FA) + \beta_4(vitamin\ D + \omega\text{-}3\ FA) + e$

where $log(ACR_4)$ represents the natural log of urine ACR at follow-up (year 4), $log(ACR_0)$ represents the natural log of urine ACR at baseline, (vitamin D) is active vitamin D treatment assignment (yes/no), (ω-3 FA) is active ω-3 FA treatment assignment (yes/no), and (vitamin D + ω-3 FA) is an interaction indicator variable for assignment to both active vitamin D and active ω-3 FA. A p value < 0.05 for β_4 will be interpreted as evidence of interaction on the multiplicative scale.

If we observe evidence of interaction on either the additive or relative scale for either study outcome, all analyses of that outcome will separately compare each of the 3 treatment groups which include at least one active study medication (i.e. cholecacliferol + placebo, placebo + ω-3 FA, and cholecalciferol + ω-3 FA) to the treatment group which receives (placebo + placebo). In contrast, if there is no evidence of interaction, as hypothesized, all participants assigned to any active vitamin D (i.e. vitamin D + placebo *or* vitamin D + ω-3 FA) will be compared to all participants assigned to no vitamin D (i.e. placebo + placebo *or* placebo + ω-3 FA). In parallel, participants assigned to any active ω-3 FA (i.e. placebo + ω-3 FA *or* vitamin D + ω-3 FA) will be compared to all participants assigned to no ω-3 FA (i.e. placebo + placebo *or* vitamin D + placebo).

VI.e. Hypothesis testing. Change in urine ACR and eGFR are continuous variables which will be tested using ANCOVA. To test the effect of vitamin D on percent change in urine ACR in the absence of interaction with ω-3 FA, we will test the following equation:

Equation 3: $log(ACR_4) = \beta_0 + \beta_1 log(ACR_0) + \beta_2(vitamin\ D) + e$

where $[\exp(\beta_2) - 1]$ represents the relative difference in percent change in albuminuria comparing active vitamin D_3 to placebo, and the corresponding 95% confidence interval and p-value reflect statistical significance of the treatment effect. To test the effect of vitamin D on change in eGFR in the absence of interaction with ω-3 FA, we will test the following equation:

Equation 4: $eGFR_4 = \beta_0 + \beta_1 eGFR_0 + \beta_2(vitamin\ D) + e$

where β_2 represents the difference in eGFR comparing active vitamin D_3 to placebo, and the corresponding 95% confidence interval and p-value reflect statistical significance of the treatment effect. Parallel equations will be used to test effects of ω-3 FA on changes in albuminuria and eGFR in the absence of interaction with vitamin D_3. An alpha level of 0.05 will be taken to represent statistical significance, without correction for multiple comparisons. We will examine the distributions of change in urine ACR and eGFR and substitute rank-sum comparisons if indicated.[152] We will test differences in proportions of participants attaining the composite dichotomous outcome using the chi^2 test.

Analyses will be performed in accordance with the intent-to-treat principle. For analyses of change in urine ACR and eGFR as continuous variables, only participants who complete study measurements at baseline and year 4 will be included in analyses (complete case analysis). Maximum effort will be made to ensure that all possible participants supply follow-up biospecimens, and all available participants will be included in continuous outcome analyses regardless of adherence to study medications. The relatively large sample size makes it likely that few or no substantial differences in baseline characteristics will be present. In the event that any baseline characteristics are not balanced between treatment groups, however, secondary analyses will add to the ANCOVA model additional terms for such baseline characteristics. If we observe differences by treatment group in medication use (e.g. glucose-lowering or antihypertensive medications) or laboratory covariates (e.g. hemoglobin A1c, CRP) over follow-up, we will explore whether such differences may mediate or obscure effects of study interventions on study outcomes by including additional covariate(s) in the ANCOVA model to assess change in β_2 (treatment effect) toward or away from zero, respectively.

VI.f. Subgroup analyses. For the vitamin D intervention, prespecified subgroup analyses will be based on baseline plasma 25(OH)D concentration, baseline urine ACR, baseline eGFR, and race/ethnicity. We hypothesize that beneficial effects of cholecalciferol on urine ACR and eGFR will be greater among

participants who at baseline (a) have low levels of plasma 25(OH)D, because vitamin D insufficiency may be particularly detrimental below a threshold vitamin D level; (b) have urine ACR ≥ 30 mg/g and/or eGFR < 60 mL/min/m^2, because persons with DKD may be particularly dependent on adequate 25(OH)D availability for renal synthesis of 1,25dihydroxyvitamin D; and/or (c) are non-Caucasian, i.e. differences in albuminuria and eGFR by race/ethnicity will narrow with vitamin D treatment. For the ω-3 FA intervention, prespecified subgroup analyses will be based on baseline levels of EPA+DHA and CRP. We hypothesize that beneficial effects of ω-3 FA on urine ACR and eGFR will be greater among participants who at baseline have (a) low levels of EPA+DHA, because ω-3 FA insufficiency may be particularly detrimental below a threshold EPA+DHA level; and/or (b) high levels of CRP, because inflammation at baseline will provide opportunity for anti-inflammatory actions of ω-3 FA. Effect modification will be tested by including interaction terms of treatment assignment with subgroup in ANCOVA models (*Equations 3 & 4*).

VI.g. *Exploratory adherence and efficacy analyses.* All primary analyses will be performed according to the intent-to-treat principle. We plan secondary, *exploratory* analyses for the following limited purposes: (a) to evaluate whether null findings, if observed, are robust (i.e. not readily attributable to defects in the study interventions or their administration); and/or (b) to more comprehensively evaluate the potential range of treatment effects, if treatment effects are observed. We understand and acknowledge that these analyses, which are based on data collected after randomization, may introduce bias into study results. We will therefore use great caution to limit their interpretation. To conduct these efficacy-based explorations, we will first repeat analyses among the subset of participants who report adherence to ≥ 80% of study medications. In addition, we will assess whether changes in 25(OH)D and EPA+DHA levels are associated with changes in urine ACR and eGFR. For the latter analyses, change in 25(OH)D concentrations or change in EPA+DHA concentration (continuous variables) will replace treatment assignment in ANCOVA models.

VI.h. *Power.* The following assumptions are made for power calculations: (1) independent and equal allocation of participants to each treatment; (2) variation in urine ACR as observed in the Multi-Ethnic Study of Atherosclerosis (MESA), including follow-up log(ACR) mean 3.085, standard deviation 1.59, and correlation with baseline 0.78; (3) variation in eGFR as observed in the Cardiovascular Health Study (CHS), including follow-up eGFR mean 70 mL/min/1.73m^2, standard deviation 25 mL/min/1.73m^2; and correlation with baseline 0.834; and (4) 20% loss to follow-up. These assumptions are conservative, in that we anticipate follow-up greater than 80% using planned participant recruiting and retention techniques, and that we will collect first-morning voids which yield lower intra-individual variation in urine ACR than the random voids collected in MESA.[153] Statistical confidence is tested using ANCOVA (*Equations 3 & 4*), with a significance level of 0.05.

Applying these assumptions, 1,500 enrolled participants (1,200 returning for follow-up) will provide 90% power to detect observed differences by treatment group of 17% in follow-up urine ACR and 2.6 mL/min/1.73m^2 in follow-up eGFR. These differences are modest compared with observed changes in urine ACR and eGFR over time in comparable MESA and CHS participants, similar to the difference in eGFR expected with the cholecalcierol intervention based on observational 25(OH)D data, and substantially more modest than the point estimate for change in urine ACR observed in our short-term cholecalciferol intervention study. Powers to detect other observed differences in the co-primary continuous outcomes are shown in **Table 2**. It is likely that an observed treatment effect, if present, will be due to a larger effect among adherent participants "diluted" by little or no effect among nonadherent participants. If adherence to study medications is 80% in each treatment group, observed differences in urine ACR and eGFR of 17% and 2.6 mL/min/1.73m^2 correspond to differences of 21% and 3.2 mL/min/1.73m^2, respectively, among adherent participants.[154] A decrease in loss to follow-up to 10% (1,350 participants returning for follow-up) will increase power to 93% for

Table 2. Study power for co-primary study outcomes, by effect size

Urine ACR		Estimated GFR	
% Difference	**Power**	**Difference (mL/min/1.73m^2)**	**Power**
15%	81%	2.0	71%
17%	**90%**	2.2	79%
19%	96%	2.4	85%
21%	98%	**2.6**	**90%**
23%	>99%	2.8	94%
25%	>99%	3.0	97%

Differences are assessed after four years total follow-up (not per year)

the detection of differences by treatment group of 17% in follow-up urine ACR and 2.6 mL/min/1.73m^2 in follow-up eGFR, while an increase in loss to follow-up to 30% (1,050 participants returning for follow-up) will decrease power to 86% for the detection of these differences. If interaction of the study interventions is present, requiring separate testing of each treatment combination against the group assigned to (placebo+placebo), we will have 80% power to detect differences in follow-up urine ACR and eGFR of 20% and 3.2 mL/min/1.73m^2, respectively, comparing any one treatment combination (e.g. active cholecalciferol + active ω-3 FA) to (placebo + placebo).

Power for the secondary composite outcome is shown in **Figure 11**, given an enrolled sample size of 1,500 participants and other assumptions listed above (including 20% loss to follow-up). Power depends on the observed 4-year incidence of the composite outcome. Based on observational data from MESA and CHS, we expect the incidences of albuminuria progression and rapid loss of eGFR to be approximately 17% each (*Section C1*), the 4-year cumulative incidence of ESRD to be <1%, and the 4-year cumulative incidence of death to be ~4.6% (cumulative incidence during the first 4 years of observation for CHS participants with self-reported diabetes), for groups assigned to placebo. The joint cumulative incidence of these outcomes will likely be at least 20-30%, yielding 80% power to detect observed relative risks of 0.69-0.76 (or lower) and 90% power to detect observed relative risks of 0.65-0.73 (or lower). A higher cumulative incidence of the composite outcome would allow detection of relative risks closer to 1.

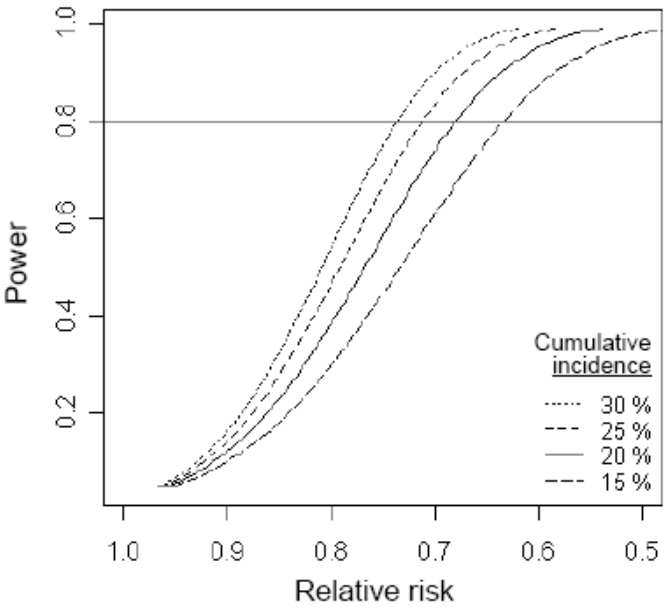

Figure 11. Power for dichotomous outcomes by relative risk (upper limit) and cumulative incidence.

It is important to acknowledge that both albuminuria and eGFR have substantial biologic variation over time, such that single measurements of albuminuria and eGFR at baseline and year 4 can lead to substantial misclassification of change in these outcomes ("noise"). This misclassification is unlikely to be biased by treatment assignment, so that it will bias results toward the null and decrease power to detect effects of study interventions. *Our power calculations are based on real-world distributions of albuminuria, eGFR, and their change over time. Thus, our power calculations account for known variability in study outcomes, with the relatively large proposed sample size of 1,500 participants essentially overcoming misclassification to permit detection of relatively modest changes in study outcomes with high levels of power.*

VII. Risks and Discomforts

VII.a. *Potential adverse effects of study interventions.* The main risks of this study arise from the interventions of the parent VITAL trial, not the VITAL Diabetes ancillary study described herein. The parent VITAL trial will test a dose of 2000 IU (40 µg) per day of vitamin D_3 (cholecalciferol) and 840 mg/d of EPA+DHA in VITAL. We believe that these doses provide the best balance of efficacy and safety, based on careful review of the literature and extensive consultation with numerous nutritional experts. We seek to obtain an adequate difference in vitamin D and EPA+DHA levels between the treatment and placebo groups to detect health benefits, and there appear to be few safety issues associated with these doses. For vitamin D, the selected dose is lower than the tolerable upper intake level (UL) for vitamin D_3 (50 µg, or 2000 IU) set by the Food and Nutrition Board of the Institute of Medicine (IOM) and the European Commission Scientific Committee on Food (EC SCF). Also, because we will exclude from the trial persons who report supplemental vitamin D intakes >800 IU/d, the selected dose ensures that no participants will be taking a supplemental vitamin D dose above 2800 IU, which is well below the 4000 IU NOAEL specified by the EC SCF. To further ensure participants' safety, we will exclude from the trial persons with a history of kidney stones, hypercalcemia, renal failure, cirrhosis, or sarcoidosis or other granulomatous disease, and will obtain calcium and PTH levels in a random subsample of participants. For fish oil, there are no specific exclusions necessary except for those currently taking the supplement and unwilling to forego use during the trial.

Potential side effects of the study agents will be assessed on each follow-up questionnaire. For fish oil, these side effects include gastrointestinal (GI) upset (presence or absence of symptoms of peptic ulcer, nausea, constipation, diarrhea); bleeding (any GI bleed, GI bleeding requiring transfusion, hematuria, easy bruising, epistaxis); skin eruptions; and physician diagnosis of atrial fibrillation or other irregular rhythms. For vitamin D, these side effects include GI symptoms as listed above, and physician diagnosis of hypercalcemia or kidney stones. Participants who self-report one of the parent VITAL trial study endpoints will be asked to sign a medical release form authorizing VITAL staff to obtain hospital/physician records. VITAL Diabetes ancillary study participants who report a kidney biopsy prior to or during VITAL participation will also be asked to sign a medical release form authorizing VITAL staff to obtain hospital/physician records. The Data and Safety Monitoring Board will compare the incidence of potential side effects in the active *v.* placebo groups for each agent, including the incidence of kidney stones with vitamin D assignment and the incidence of GI symptoms, skin abnormalities, and bleeding with fish oil assignment.

VII.b. *Psychosocial risks.* There is also the possibility of social/psychological risk that could result from inadvertent disclosure of confidential information from the questionnaires or blood tests. However, we have many safeguards in place to avoid this possibility, and the experienced team leading the parent VITAL trial has never had an inadvertent breach of confidentiality in prior trials. Each name on the mailing tape will be assigned a study identification number and sent a package containing: a letter explaining the rationale for VITAL and what participation would entail; an informed consent form; and a brief questionnaire with items on demographics; medical history; current use of supplements containing vitamin D or fish oil; current use of other supplements or medications; dietary intake of vitamin D and consumption of fish; cancer and vascular risk factors; and potential effect modifiers. Those who are initially willing and eligible will be entered into the 3-month run-in phase. Those who remain willing and eligible, and who are compliant with pill-taking during the run-in phase, will be randomized into the parent VITAL trial. Participants who are additionally eligible for the VITAL Diabetes ancillary study proposed herein will be additionally entered into this ancillary study.

The potential risk of disclosure of confidential information is guarded against by maintaining completed questionnaires and blood test results in locked files accessible by authorized personnel only. In these files, participants are identified only by study ID. Subject identifiers are stored in separate computer files with password protection, and results will be presented only in the aggregate. In addition, employees involved with the proposed study will be asked to sign a form agreeing not to disclose any information to which they might have access, regardless of their personal perception about its confidentiality. Each employee of the study with human subject contact participates in an institutional (Brigham and Women's Hospital) human subjects educational program, which consists of reviewing regulatory and informational documents pertaining to human subjects research; passing a test on ethical principles and regulations governing human subjects research; and signing a statement of commitment to the protection of human subjects. Finally, all such employees are required to participate in HIPAA training.

VIII. Potential Benefits

VIII.a. *Potential Benefits to Participants.* For the majority of participants, there will be few direct benefits from participating in this primary prevention study other than the awareness of being involved in a large endeavor to answer relevant and timely questions regarding the possible benefit of vitamin D and fish oil. During the trial itself, we will receive inquiries from the participants regarding both specific and general health concerns. We will respond to each of those questions, primarily directing participants to published sources or recommending that they see their local health provider who is familiar with their medical history.

VIII.b. *Potential Benefits to Society.* The potential benefits to society relate to the increasing use of both vitamin D and fish oil, with data not yet clearly indicating efficacy in the general population with respect to prevention of cancer and CVD (parent VITAL trial outcomes) or with respect to albuminuria and glomerular filtration rate (VITAL Diabetes ancillary study outcomes). Such data will help guide individual decisions, clinical recommendations, and public health guidelines.

IX. Monitoring and Quality Assurance

An independent Data and Safety Monitoring Board (DSMB) has been assembled for the parent VITAL trial, consisting of experts in clinical trials, epidemiology, biostatistics, relevant clinical areas of cancer and CVD, and NIH representatives. The DSMB will annually examine the progress of the trial and the unblinded data on study endpoints and possible adverse effects to recommend continuation, alteration of study design, or early termination, as appropriate. Interim trial results will be assessed with the Haybittle-Peto rule,[155, 156] adjusting for multiple looks. In this method, interim results are compared to a z-score of 3 standard deviations (p=0.0027) throughout the trial. The final results may then be interpreted as having close to nominal significance levels. This rule appropriately requires very strong evidence for early stopping, is more conservative than the Pocock[157] and O'Brien-Fleming[158] rules and the alpha-spending function,[159] and can be conducted at convenient times without inducing statistical complexity.[160]

While these rules are intended for the primary endpoints, the goal of VITAL is to assess the overall balance of benefits and risks of the two agents in the primary prevention of cancer and CVD. Thus, consideration will also be given to the secondary endpoints that are needed in the interpretation of overall results. In addition, the monitoring rules will serve solely as guidelines in decisions regarding continuation or stopping of treatment arms. All decisions must be made after examining the totality of evidence, including other trial data, on these agents.

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Randomized Clinical Trial of Vitamin D and Omega-3 Fatty Acids for Diabetic Kidney Disease

Protocol Addendum

Changes to analytic plan
Submitted in NIH grant renewal application 3/2/2015
Implemented upon funding 2/24/2016

15. Data analysis

15a. Descriptive analyses. We will describe creation of the VITAL diabetes sub-cohort according to CONSORT guidelines, including numbers of participants who consent to VITAL participation; report a diagnosis of diabetes; are approached for the DKD ancillary study; enroll in the DKD ancillary study; and complete year 2 and 5 year biosample collections. We will document reasons that participants do not complete the proposed studies. Among participants recruited into the DKD ancillary study, we will compare baseline characteristics of those who do and do not return at least one follow-up biosample collection. Among participants who return a follow-up biospecimen, we will describe by treatment assignment baseline characteristics and changes in clinical characteristics over the course of the study.

15b. Study outcomes. With an extension of follow-up from 2 to 5 years, we will have sufficient time to effectively evaluate change in estimated GFR (eGFR), a clinically relevant outcome.[175-179] Therefore, if this competitive renewal is funded, change in eGFR from baseline to year 5 will supplant 2-year changes in albuminuria and eGFR as our primary study outcome. Change in eGFR will be calculated and summarized by treatment group as $[eGFR_5 - eGFR_0]$, where $eGFR_5$ represents eGFR at follow-up (year 5) and $eGFR_0$ represents eGFR at baseline. As secondary outcomes, we will examine changes in urine ACR from baseline to years 2 and 5, time to the development of rapid eGFR loss (loss of eGFR ≥40% from baseline to year 2 or 5),[175-179] and time to a composite of rapid eGFR loss, end stage renal disease (maintenance dialysis or kidney transplant verified by medical records review), or death. The composite outcome addresses large changes in eGFR which have particularly high clinical importance and reduces the potential impact of competing risks that preclude assessment of eGFR at follow-up.

15c. Intention to treat and missing data. Analyses will be performed in accordance with the intent-to-treat principle, meaning that all participants who return at least one follow-up biosample will be included in analyses, regardless of adherence or other characteristics observed after randomization. Multiple imputation for study participants missing year 5 eGFR will be conducted using chained equations, utilizing baseline data as well as eGFR and urine ACR data available at year 2.[180] Results from 10 imputation datasets will be combined using Rubin's rules.[181]

15d. Treatment interaction. We will assess for interaction of the study interventions (vitamin D and omega-3 fatty acids) for the primary outcome.[182] We will use a linear mixed model to test for interaction:[183]

$$\textit{Equation 1: } eGFR_{ij} = \beta_0 + \beta_1(\text{vitamin } D_i) + \beta_2(\omega\text{-3 FA}_i) + \beta_3(\text{vitamin } D_i \times \omega\text{-3 FA}_i) + \beta_4(\text{vitamin } D_i \times t_{ij}) + \beta_5(\omega\text{-3 FA}_i \times t_{ij}) + \beta_6(\text{vitamin } D_i \times \omega\text{-3 FA}_i \times t_{ij}) + a_i + e_{ij}$$

where i denotes the ith participant and $j=0,2,5$ represents the time period, t is time modeled categorically, (vitamin D) is active vitamin D treatment assignment (yes/no), (ω-3 FA) is active omega-3 treatment assignment (yes/no), a_i is a random effect for patient, and ε is statistical error. A p value < 0.05 for β_6 will be interpreted as evidence of interaction. If we observe interaction, all analyses will separately compare each of the 3 active treatment groups to the treatment group which receives (placebo + placebo). If there is no evidence of interaction, as hypothesized, all participants assigned to any active vitamin D will be compared to all participants assigned to placebo vitamin D, and all participants assigned to any active omega-3 fatty acids will be compared to all participants assigned to placebo omega-3 fatty acids.

15e. Hypothesis testing. The primary outcome is a continuous variable which will be tested using a linear mixed model with random intercepts. To test whether the change in eGFR differs by vitamin D treatment assignment in the absence of interaction with omega-3 fatty acids, we will test:

$$\textit{Equation 2: } eGFR_{ij} = \beta_0 + \beta_1(\text{vitamin } D_i) + \beta_2(t_{ij}) + \beta_3(\text{vitamin } D_i \times t_{ij}) + a_i + e_{ij}$$

where β_3 represents the difference in change in eGFR over time, comparing those on active vitamin D_3 to those on placebo. The corresponding 95% confidence interval and p-value reflect statistical significance of the treatment effect. We will use this model to describe treatment effects at years 2 and 5 but reserve statistical inference for year 5 alone. Corresponding equations will test the effect of omega-3 treatment assignment on eGFR as well as treatment effects on urine ACR, analyzed as a log-transformed continuous variable. The relatively large sample size makes it likely that few or no substantial differences in baseline characteristics will be present. In the event that any baseline characteristics are not balanced between treatment groups, sensitivity analyses will add to the model additional terms for such baseline characteristics. If we observe differences by treatment group in medication use (e.g. glucose-lowering or antihypertensive medications) or

laboratory covariates (e.g. hemoglobin A1c, CRP, IL-6) over follow-up, we will explore whether such differences may mediate or attenuate treatment effects by adding terms for these covariates to the model. We will assess secondary categorical outcomes using discrete Cox proportional hazards models.[184]

15f. Subgroup analyses. For the vitamin D intervention, prespecified subgroup analyses will be based on baseline total and bioavailable serum 25(OH)D concentrations, urine ACR, and eGFR as well as race/ethnicity: we hypothesize that effects will be greater among participants who have low 25(OH)D at baseline, who have DKD (urine ACR ≥ 30 mg/g or eGFR < 60 mL/min/m^2) at baseline, or are Caucasian.[185,186] For the omega-3 fatty acid intervention, prespecified subgroup analyses will be based on baseline levels of EPA+DHA and hsCRP: we hypothesize that effects will be greater among participants who low levels of EPA+DHA or high concentrations of hsCRP at baseline. Effect modification will be tested by including interaction terms of treatment assignment with subgroup in linear mixed models (*Equation 2*).

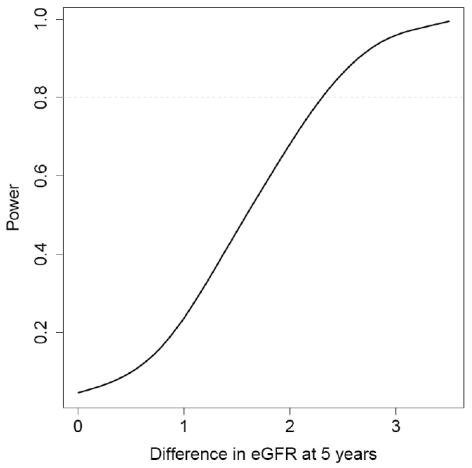

Figure 7. Study power by effect size for the primary outcome (difference in estimated GFR in mL/min/1.73m^2).

15g. Exploratory adherence and efficacy analyses. All primary analyses will be performed according to the intent-to-treat principle (Section D15e). We plan secondary, ***exploratory*** analyses for the following limited purposes: (a) to evaluate whether null findings, if observed, are robust (i.e. not readily attributable to defects in the study interventions or their administration); and/or (b) to more comprehensively evaluate the potential range of treatment effects, if treatment effects are observed. We understand and acknowledge that these analyses, which are based on data collected after randomization, may introduce bias into study results. We will therefore use caution to limit their interpretation. We will first repeat analyses among the subset of participants who report continuous adherence to ≥ 80% of study medications. In addition, we will assess whether changes in 25(OH)D and EPA+DHA levels are associated with changes in urine ACR and eGFR. For the latter analyses, change in 25(OH)D concentrations or change in EPA+DHA concentration (continuous variables) will replace treatment assignment in linear mixed models.

D16. Power. We calculated power using simulation (2000 replications) and the following assumptions: independent and equal allocation of participants to each treatment; 1,058 participants (80%) contributing at least one follow-up blood sample (as supported in Section D10); variation in eGFR and its change over time as observed in the preliminary data from our study population (Section D12); and no interaction between treatments. With these assumptions, we have 80% power (two-sided alpha=0.05) to detect a 2.3 mL/min/1.73m^2 difference in eGFR at year 5, comparing each active treatment to placebo. Power for alternative effect sizes is shown in **Figure 7**. We do not present power for secondary outcomes because the incidence of these outcomes is difficult to predict with available data.