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MULTIFACETED NUTRITIONAL INTERVENTION IN HEMODIALYSIS PATIENTS

by

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DISSERTATION

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DEDICATION

This dissertation is dedicated to the significant people in my life:

Nadiah, Muhammad Irfan & Nabeeha
My beloved wife who pouring hours of work in loving our children, and lent me emotional assistance during ups and downs of PhD life – enabled me to dedicate hours of work, contemplation and writing necessary to complete this research.

Hj Mat Daud, Hjh Che Azizah, Hj Muslal, Hjh Masnah
My beloved parents and parents in law who never failed to lend me moral and financial support and never forget to pray for my success every day and night.

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My dearest brothers and sisters who always inspired me to be a better person.

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	:	Amino acid
ApoA1	:	Apolipoprotein A1
Alb	:	Albumin
BMI	:	Body Mass Index
CETP	:	Cholesteryl ester transfer protein
CKD	:	Chronic kidney diseases
CRP	:	C-reactive protein
CVD	:	Cardiovascular diseases
eGFR	:	Estimated GFR
ESRD	:	End stage renal/kidney disease
GFR	:	Gomerular filtration rate
Hb	:	Hemoglobin
HD	:	Hemodialysis
HDLC	:	High density lipoprotein cholesterol
IL-6	:	Interleukin-6
IQR	:	Interquartile ranges
KDIGO	:	Kidney Disease: Improving Global Outcomes
KDOQI	:	Kidney Disease Outcomes Quality Initiative
LDLC	:	Low density lipoprotein cholesterol
MDA	:	Malondialdehyde
MICS	:	Malnutrition-inflammation complex syndrome. Please see (*).
MIS	:	Malnutrition Inflammation Score

MVDA	:	Multivariate data analysis
NMR	:	Nuclear magnetic resonance
PCA	:	Principal component analysis
PEW*	:	Protein energy wasting. This is being used interchangeably with malnutrition and MICS
PLS	:	Partial least square
PLS-DA	:	Partial least square – discriminant analysis
pmp	:	Per million population
RCT	:	Randomized controlled trial
RRT	:	Renal replacement therapy
SCr	:	Serum creatinine
SD	:	Standard deviation
SEM	:	Standard error
SREBPs	:	Sterol regulatory element binding protein
SR-B1	:	Scavenger receptor class B type I
TAG	:	Triacylglycerol
TAP	:	Total antioxidant power
TC	:	Total cholesterol
TNF-α	:	Tumor necrosis factor alpha
TP	:	Tocopherol
TRF	:	Tocotrienol-rich fractions
TT	:	Tocotrienol
USRDS	:	US Renal Data System

WHO   :   World Health Organization

wk   :   week

CHAPTER I

INTRODUCTION

Background

Chronic kidney disease (CKD) has emerged as one of the major public health issues worldwide. In the United States, it has been estimated that 14% of the adult population suffers from CKD (defined by an estimated glomerular filtration rate of $<60ml/min/1.73m^2$) [1]. Moreover, 580, 741 patients in the US are already reaching the end stage of renal disease (ESRD) (1752 per million population) and an additional 200,000 people will develop ESRD in the next 10 years [2]. Once diagnosed with ESRD, one needs to undergo renal replacement therapy (RRT) as a life-saving treatment. The most common RRT modality in the United States is hemodialysis (HD) which account for 98% of total ESRD patients on dialysis [1].

ESRD patients experience alarmingly high rates of cardiovascular complications. Cardiovascular diseases (CVD) account for 50% of mortality in ESRD patients which is 15-30 times higher than the age-matched general population [3-5]. Pathophysiology of CVD in this population is complex as it involves traditional CVD risk factors as well as non-traditional risk factors specific to the uremic population. It is well known that dialysis patients experience chronic inflammation due to various dialysis and non-dialysis factors. Because the dialysis procedure forces one's blood through a dialyzer membrane, blood contact with a nonbiocompatible membrane or with non-sterile dialysate solutions and an impure dialysate triggers the inflammatory process [6]. However, dialysis factors themselves may not be the major factors for instigating the inflammatory process, because CKD patients not yet on dialysis also have high a incidence of inflammation [6-8].

In addition, another comorbid condition that is highly prevalent in ESRD patients is protein-energy wasting (PEW), which is by far the strongest predictor for adverse outcome and mortality [9]. PEW is characterized by a loss of muscle mass, unintentional weight loss and a significant decline in nutritional parameters such as albumin and prealbumin [10]. Low serum albumin (hypoalbuminemia) in this population is partly due to inadequate energy and protein intake, loss of amino acid/protein during dialysis, and/or volume overload as a result of non-compliance to fluid restriction. Because albumin contains thiol moieties, which is the most important antioxidant in the plasma, low plasma albumin correlates with diminished plasma total antioxidant capacity which provides a pathophysiological link to cardiovascular mortality in ESRD patients with hypoalbuminemia [11]. Furthermore, PEW and inflammation often occur concomitantly in HD patients (referred together as the malnutrition-inflammation complex syndrome, MICS). As a matter of fact, persistent inflammation is responsible for a cascade of reactions that causes an increase in resting energy expenditure, loss of muscle mass and oxidation [12] which eventually exacerbates PEW.

Oxidative stress, defined as an imbalance between the pro- and anti-oxidant system, is highly elevated in HD patients as compared to healthy-matched control [11, 13, 14]. Impaired oxidative balance in HD patients is partly attributed to increased reactive oxygen species production, reduced clearance, and a poor antioxidant defense system [14]. Oxidative stress is linked to CVD because of its involvement in oxidative modification of low-density lipoprotein (LDL). Oxidized LDL instigates a cascade of reactions including adhesion of circulating monocytes on endothelial cells, migration of monocytes into the arterial intima and platelet activation, and expression of tissue factor by endothelial cells [14].

Furthermore, advanced CKD patients including ESRD on dialysis also develop dyslipidemia characterized by impaired synthesis and activity of high-density lipoprotein (HDL) and delayed catabolism of triglyceride-rich apo B containing lipoprotein, which subsequently leads to elevated triacylglycerols (TAG) and low plasma HDL cholesterol (HDLC) [15]. Accumulating evidence indicates that the concentration of plasma apo-A1 and lecithin-cholesterol acyl-transferase (LCAT) are decreased [16, 17] which in turn impedes HDL-mediated reverse cholesterol transport [18], a process for disposing excessive cholesterol from extra hepatic tissues and blood vessel walls. As oxidative stress is highly prevalent in ESRD patients, byproducts of oxidation reactions can be involved in oxidative modification of lipoproteins which consequently can disrupt the protective effects of HDL against atherosclerosis [18]. Taken together, a combination of MICS coupled with oxidative stress and dyslipidemia is highly conducive to exacerbating atherosclerosis in the HD population.

Rationale for the current project

Although much knowledge have been gained in understanding the complex pathophysiology of CVD in this population, multifaceted clinical interventions aimed at improving CVD outcome in HD patients have been marginally successful. This is evident with from the fact that two thirds of dialysis patients die within five years of commencing dialysis predominantly related to CVD [19] despite substantial improvement in dialysis therapy. In fact, mortality rates in ESRD patients in the US (>20% per year), is worse than many fatal cancers [1, 20]. Some major clinical trials that failed to demonstrate beneficial results of improving mortality and CVD outcomes include AURORA [21], 4D trials [22] (both studies used lipid lowering therapy approach) and HEMO trial (increased dialysis dose)[23, 24]. Interestingly, two clinical trials that used 'dietary intervention' as a mean to improve cardiovascular outcome showed promising results. Off the

two, one study – 'Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE)' demonstrated that supplementation with 800IU of vitamin E per day resulted in reduction of overall mortality and morbidity based on composite cardiovascular end points [25]. Additionally, a recent observational study in 1046 diabetic hemodialysis patients demonstrated that patients with the lowest tertiles of plasma α-tocopherol had a 79% higher risk of stroke and 31% higher risk of all-cause mortality compared to those with the highest tertiles [26], which exemplifies the role of maintaining adequate dietary vitamin E/antioxidant intake. Taken together, from the nutritional stand point, an effective yet a safe therapeutic approach is needed to reduce comorbid conditions in this population. Nevertheless, dietary strategies to correct these problems have been inconclusive.

Recommendation for Omega-3 fatty acids studies in HD population stemmed from the fact that these nutrients have been shown to possess anti-inflammatory, anti-oxidative and lipid altering properties *in vivo*, *in vitro* and also in a clinical setting [27-30]. Furthermore, the fact that HD patients have lower plasma and erythrocytes levels of omega-3, (among the lowest as compared to the other clinical population [31]) related to dietary monotony [32] and a high inflammatory and oxidative milieu, exemplify the needs for dietary intervention studies. However, current level of evidence is still controversial to mandate incorporation of this nutrient into standard nutritional care of HD patients as a mean to prevent CVD.

Similarly, vitamin E tocotrienols (TT) recently have received great scrutiny due to their superior biological activity and therapeutic benefits as compared to tocopherols (TP) [33-35]. TT have been reported to confer antioxidant protection from oxidative damage [36], have anti-inflammatory properties [37] and are potentially cardioprotective due to their lipid altering properties [38]. Taken together, the fact that ESRD patients undergoing HD are at high risk for

cardiac mortality related to widely prevalent malnutrition, inflammation, heightened oxidative stress and dyslipidemia, therefore, dietary intervention to evaluate the effectiveness of these nutrients to reduce the aforementioned co-morbid conditions is recommended [6].

However, the global impact of such dietary intervention in a diverse clinical population is difficult to interpret, partly due to 1) inter-individual variation in the response against dietary intervention; 2) the existence of various confounding factors such as the difference in underlying comorbidities, medication profile, genetic makeup and socioeconomic disparities; 3) lack of effective approach to accurately account for compliance/non-compliance towards the intervention and 4) the nature of traditional biomarkers that are unable to provide a complete picture to reflect the effect of such intervention. With the emergence of 'omics' technology, a comprehensive systematic and simultaneous profiling of the complete set of metabolites is now possible [39, 40]. Given that, metabolomics approach has been used in various fields including investigating the postdose drug effects *vs* predose metabolic profiles [41], studying drug toxicity and gene function [42], evaluating the progression of disease [43] and determining changes in metabolite excretion profiles following dietary changes [39], we therefore sought to apply metabolomics to understand a broader spectrum regarding the effects of these nutrients in HD patients.

Specific aims of the current project

The objective of this study was to investigate the effects of supplementation with nutrients having anti-inflammatory and antioxidant properties on nutritional, lipids, inflammatory and oxidative status. The central hypothesis of the present study was that supplementation with protein as an anabolic nutrient; omega-3 fatty acids and tocotrienol rich fraction (TRF) as anti-

inflammatory and antioxidant nutrients; will improve nutritional status, lipid profiles, as well as inflammatory and oxidative stress markers. The rationale for the proposed studies was that once the effects of these nutrients are established, larger clinical trials can be performed to establish clinical evidence in order to accommodate incorporation of these nutrients into dietary regimen of HD patients as an adjunct therapy to attenuate co-morbid conditions in these patients. Accordingly, the following specific aims were established;

Specific aim 1: To document the global effects of protein and omega-3 supplementation on nutritional status and inflammatory markers in chronic hemodialysis patients.

Specific aim 2: To document the global effects of TRF supplementation on nutritional indices, inflammatory markers, oxidative status and lipid profiles in chronic hemodialysis patients.

Specific aim 3: To determine changes in metabolomics profiles following TRF supplementation.

CHAPTER II

REVIEW OF LITERATURE

Overview of kidney structures and functions

The kidneys are a pair of bean shape organs located retroperitoneally between the transverse process of T12-L3 vertebrae, with the left kidney located on a slightly superior position than the right kidney. Each kidney weighs ~ 150g and 135g in males and females, respectively. Despite representing less than 1% of total body weight, the kidneys receive approximately 20% of the cardiac output, which is the highest blood flow of all the larger organs in the body, filtering ~ 1600 L of blood/day. The blood supplies for the kidneys originate from the paired renal arteries at the level L2 of the vertebrae. Each kidney comprised of more than a million functional units – called nephrons. Each nephron consists of different functional segments with a typical cellular appearance; the renal corpuscle (glomerulus and bowman capsule), proximal convoluted tubule, descending loop of Henle, ascending limb, distal convoluted tubule and the collecting duct [44, 45].

The kidneys serve important functions, including maintaining homeostatic balance with regards to fluids, electrolytes, organic solutes and acid-base balance. In the renal corpuscle, perfusion pressure of the glomerulus forces molecules smaller than 6500Da to pass through semipermeable membrane of the glomerulus into Bowman capsule as filtrate. About 80% of the blood entering glomerulus returns to the blood stream while the remaining 20% become filtrate. However, 99% of the filtrate will be reabsorbed via renal tubules and only 1% of the filtrate is excreted as urine. This process is achieved via a unique structure of renal tubules with varying

permeability between segments and hormonal regulation allowing the kidney to produce a final urine with diverse concentration of electrolytes, osmolality, pH and volume [45].

Kidneys also play substantial roles (not related to excretion) as in the regulation of blood pressure that is achieved via renin-angiotensin mechanism. Granular cells of the juxtaglomerular apparatus in the kidneys secrete renin which catalyzes the formation of angiotensin I from a plasma globulin, angiotensinogen. Angiotensin I is further converted into angiotensin II, a strong vasoconstrictor causing constriction of the blood vessels and subsequently increased blood pressure. Angiotensin II also contributes to the increase in blood volume by increasing water and sodium reabsorption via stimulation of aldosterone hormone [44, 46].

Moreover, renal corticointerstitial cells of the kidneys produce erythropoietin (EPO), which plays a vital role in maturation of erythrocytes in the bone marrow and other non-homopoietic roles such as a neuroprotective agent in the response to neuronal injury in the brain [47] and in the wound healing process mainly by improving vascular perfusion [48]. Another function of the kidney includes the maintenance of calcium-phosphorus homeostasis. This is achieved via a complex interplay of various effector organs (the guts, kidney and bone) and hormones (parathyroid hormone, calcitonin and active vitamin D) – the kidney role in this process includes activation of vitamin D and excretion of phosphorus and calcium [45]. The diverse role of kidney is summarized in **Table 2-1**.

Chronic kidney disease and end-stage renal disease

Definition

Chronic kidney disease (CKD) is defined as progressive, irreversible abnormalities in kidney structure and function, present for more than three months which has implication for health [49]. Based on the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines, CKD is

classified based on cause, glomerular filtration rate (GFR) category and albuminuria category [49]. Given the difficulties to accurately measures GFR in clinical settings, it is often estimated using mathematical calculation based on serum creatinine levels, demographic and anthropometric parameters [50]. Based on the estimated GFR (eGFR) patients with CKD are classified from G1 (normal/reduced kidney functions) to G5 (kidney failure). However, it should be noted that decrease in GFR alone is not an absolute criterion for kidney damage diagnosis but rather should be manifested by either pathological abnormalities or markers of kidney damage such as abnormalities in imaging test and composition of the blood and urine [49, 51]. Among individuals with CKD, one is typically considered as end-stage renal disease (ESRD) when GFR is severely reduced to <15ml/min/1.73 m^2 (**Table 2-2**) and this is accompanied with clinical symptoms mandating the need for lifelong renal replacement therapy (RRT) to maintain survival.

Incidence and Prevalence

In the United States incident of end-stage kidney disease rose steadily since 1980 to 2001, but has leveled off since then to approximately 350 people per million population (pmp) [1]. When these data are compared to other well developed nations, ESRD incidents in the US range from twice to thrice as much of those in the United Kingdom, France, Denmark, Russia, New Zealand, Australia and most European countries (**Figure 2-1**).

In term of ESRD prevalence, United States continues to report one of the highest rates (1870 pmp), just behind Taiwan (2584 pmp) and Japan (2260 pmp). In contrast, other affluent countries such as the United Kingdom, Sweden, France and Australia have reported prevalences rate twice as low as in the US [1].

Etiology

Uncontrolled underlying chronic diseases such as Diabetes Mellitus (DM) and hypertension are the most common causes of ESRD. Based on the United States Renal Data System, 41% of incident ESRD cases are due to DM (718 cases pmp), follow by hypertension (477 cases pmp), glomerulonephritis (274 cases pmp) and polycystic kidney diseases (92 cases pmp). Internationally DM is reported as a primary cause of ESRD in more than 60% of the new ESRD patients in Mexico, Singapore and Malaysia, while Russia, the Netherland, Norway and Romania reported rates below 20% [1]. Other rare causes include autoimmune disease (especially systemic lupus erythematous associated nephritis), tubular interstitial nephritis, drug toxicity and glomerular diseases caused by hepatitis B, C and HIV viruses.

Uncontrolled DM contributes to functional changes in glomeruli including hyperfiltration and hyperfusion, subsequently leading to histological alteration of the nephron, increasing capillary permeability to macromolecules characterized by micro and macroalbuminuria (>300mg/day). This is accompanied by structural changes including glomerular basement thickening and hypertrophy, as well as mesangial expansion leading to glumeroslosclerosis and tubulointerstitial fibrosis. Metabolic processes such as hyperglycemia and hormonal mediators including glucagon and growth hormones are implicated in diabetic nephropathy, over time, leading to overt proteinuria, azotemia and culminating in ESRD [52].

Treatment Modalities

Patients with advanced CKD (stage G4/5) may eventually deteriorate to the degree that the remaining kidney functions are unable to keep them off from significant kidney failure symptoms. Some of the severe symptoms include excessive fluid overload leading to congestive

heart failure, severe nausea, vomiting, confusion/decrease alertness and seizures. Moreover, certain electrolytes such as sodium, potassium or bicarbonate could be dangerously elevated, affecting functionality of the organ. This is commonly occurring when eGFR is $<15ml/min/1.73m^2$, however some patients might be more symptomatic at a higher eGFR [46]. Therefore, RRT is needed at this point to limit clinical symptoms and to maintain their survival. There are several options of RRT available including different forms of dialysis (e.g. hemodialysis, peritoneal dialysis) and kidney transplantation. In-center hemodialysis (HD) is the most common type of treatment modality in the US accounting for 98% of the total dialysis patient population [1].

In HD procedure, patient's blood is pump through semipermeable membrane dialyzer in the extracorporeal circuit, while dialysate (solution containing sterilized mineral ions, bicarbonate and glucose [53]) is flowing in the opposite direction of the blood flow (**Figure 2-2-B**). Removal of waste solutes and fluid is achieved by the principle of hydrostatic pressure and diffusion across the concentration gradient. Because HD required permanent access to blood stream during each treatment, vascular access either via an arteriovenous fistula, arteriovenous graft or intravenous catheter has to be created. Patients undergoing in-center chronic HD treatment typically attend the clinic for three times a week for three to four hours per treatment. During HD procedure, blood is drawn out from the access route at a rate of 200-400 mL/min while patient is closely monitored for changes in blood pressure.

Meanwhile, peritoneal dialysis use similar principle as HD, except the fact that it uses the patient's own peritoneum membrane to remove waste solute and fluids (**Figure 2-2-A**). In this process, a dialysate fluid is introduced into the abdominal cavity through a permanent catheter implanted in the abdomen. This dialysate fluid (containing sterile mineral ions,

bicarbonate and glucose) remains in the patient's abdominal cavity (4-6 hours) to allow waste solutes to exchange across the concentration gradient before removal and replacement with new ones. This process is repeated at least four times a day for patients undergoing continuous-ambulatory peritoneal dialysis treatment.

Kidney transplantation (**Figure 2-2-C**) offers a better prospect as compared to dialysis provided that the transplanted kidney functions successfully. However, RRT is still dominated by HD in large due to shortage of donor kidneys [54]. In this procedure, a compatible kidney from living/deceased donor is transplanted into the patient's pelvis and connection is made into the artery and vein, including surgical incision of the transplanted ureter into the bladder. Patient receiving a kidney transplant needs to also rely on immunosuppression drugs to avoid rejection.

Complications

In CKD, as GFR is decreased the capacity to respond to the intake of minerals, water and other solutes become less flexible due to reduced number of functional nephrons, leading to the accumulation of solutes and water in the body. Compounds that are normally being excreted such as phosphates, sulfates, uric acids and hydrogen ions are subsequently retained in the body as the disease progresses. Retention of hydrogen ions results in metabolic acidosis, a clinical disturbance characterized by increased plasma acidity that could lead to disturbance of several organs system. In addition, toxic compounds such as phenols, guanidine, organic acids, indols, polyamines β2-microglobulin and some other trace elements will accumulate leading to hormonal deficiency and the inability to respond to stimuli (e.g. insulin resistance, eryhthropoietin resistance) [46].

Accumulation of blood urea and other toxic compounds that normally are being excreted by the kidney could leads to uremia. Uremia is a clinical syndrome due to imbalance of body fluids, electrolytes, hormones and metabolic abnormalities that occur following dysfunctional kidneys [46]. Some common signs and symptoms of uremia includes neural and muscular manifestation (e.g. fatigue, peripheral neuropathy, decrease mental acuity, anorexia and nausea, restless legs, cramps, decrease sense of smell and taste), endocrine and metabolic affects (amenorrhea, reduce body temperature, increased protein-muscle catabolism), while severe untreated uremia could lead to seizures, coma and cardiac arrest [54]. Uremia could also lead to oxidative stress and oxidation of macromolecules as evident by increased levels of reactive-oxygen species (ROS), oxidized plasma proteins and lipids [55].

Anemia (as defined by hemoglobin levels $\leq$ 13g/dL for men and $\leq$ 12 g/dL for women [32]) is another common complication of ESRD. Hemoglobin <11 g/dL are often associated with lower quality of life and increased cardiovascular mortality and morbidity [56]. Anemia in CKD and ESRD patients has multifactorial physiopathology but it is generally accepted that it may occurs due to the decreased in erythropoietin production as result of the loss of functioning renal tubular mass. In dialysis patients, protein-energy malnutrition and chronic infection lead to erythropoietin resistance and anemia [57]. Inflammation could also affect mucosal absorption of ferum and trigger gastric bleeding which further exacerbates anemia in ESRD [58]. Anemic condition leads to prolonged fatigue, decreased immune functions and increased workload of the heart resulting in ventricular hyperthrophy, a risk factor for CVD[59]. Chronic anemic patients also experience fluid and sodium retention, high cardiac output and reduced oxygen carriage in the circulation [60]. Moreover, anemia could also induce a series of cardiovascular and neurohormonal compensations including increased plasma norephineprine, renin activity,

aldosterone, growth hormone and arterial natriuretic peptide that could have injurious cardiovascular complications in long-term [60].

Furthermore, ESRD patients often experience bone and mineral disorders characterized by hyperphosphatemia, hyperparathyroidism and systemic deficit in activation of vitamin D. A cascade of changes in the metabolism of calcium, phosphorus and vitamin D occurs following reduction of GFR leading to these disorders. As GFR is reduced, activity of 1α-hydroxylase enzyme declines, causing impaired conversion of vitamin D into its active form and subsequently limiting calcium absorption from the intestinal tracts. On the other hand, phosphorus levels in the blood are increased due to decreased renal tubular functions. Because vitamin D is also needed in suppression of the production of parathyroid hormones (PTH), reduced vitamin D levels (the active form) together with reduced serum calcium lead to overproduction of PTH in a counteregulatory action to release calcium from the bones store [61]. Ironically, increased in serum phosphorus levels (hyperphosphatemia) further exacerbate PTH production leading to hyperparathyroidism.

Although dialysis is an effective treatment to alleviate the adverse effect of uremia, some residual uremic toxins remain [62]. This is evidenced by high mortality rate, poor quality of life and high hospitalization rates in patients undergoing dialysis despite advancement in dialysis technology. It has been estimated that more than 20% of ESRD patients undergoing HD treatment die due to uremia and treatment complications [20].

ESRD main complication: cardiovascular diseases

Cardiovascular disease (CVD) is a major complication affecting CKD and ESRD population. It has been estimated that cardiovascular death accounts for more than 50% of mortality in ESRD

patients undergoing HD treatment [19]. Common forms of CVD occurring in this population include acute myocardial infarction and several forms of atherosclerotic vascular diseases (e.g. chronic coronary artery disease, stroke and transient ischemic attacks) [5].

Atherosclerosis is an inflammatory process initiated by several factors including accumulation of LDLs in the artery wall [63, 64]. When plasma LDL levels exceeds certain threshold, they enter artery faster than they can be removed leading to their accumulation [63]. Because accumulated LDLs are prone to oxidation, the oxidative stress milieu in ESRD patients exaggerates the acceleration of atherosclerosis in part by modification/oxidation of LDLs [64]. The modified LDLs trigger endothelial cells to express a protein known as monocyte chemotactic protein-1 (MCP-1), which in turn attracts monocytes into the arterial wall and promotes their differentiation to macrophages. Macrophages engulf oxidized LDLs via their scavenger receptor and express several cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) which activates endothelial cells to express adhesion molecules (e.g. E-selection, VCAM-1 and ICAM-1). Formation of lipid-filled foam cells following macrophages' ingestion of LDLs and the binding of monocytes to the endothelium by adhesion proteins marks the beginning phase of atherosclerosis [63].

Evidence from autopsy and clinical investigation has demonstrated that coronary artery plaques are highly prevalent in patients with advanced kidney disease [64, 65]. **Figure 2-3** summarizes the atherogenesis/ coronary artery plaque development processes. As reviewed by Stenvinkel et al [64], during the early phase of atheroma, prolonged inflammatory conditions and persistent dyslipidemia result in increased susceptibility to oxidation of lipids and other macromolecules. Fatty streak is developed following recruitment of inflammatory cells and the accumulation of foam cells. Degradation of extracellular matrix occurs when the activated

leukocytes secreted proteinase, and proinflammatory cytokines stimulated T helper cell type 1 (Th1) which could limit the synthesis of new collagen, thinning the fibrous cap and making it susceptible to rupture. When the rupture occurs, coagulation of the blood on the plaque caused the formation of thrombus and manifested as an acute coronary syndrome [64].

Various factors have been postulated to be involved in the increased cardiovascular burden in ESRD as illustrated in **Figure 2-4**. Briefly, ESRD patients undergoing chronic HD are exposed to various conditions that increase the cardiovascular diseases burden including 1) underlying co-morbid conditions such as diabetes; 2) uremic residuals which triggers inflammatory modulation, heightened oxidative stress and influences appetite and food intake; 3) dialysis technique related factors that could worsen inflammatory responses and nutrient losses; 4) undesirable outcome of dietary restrictions (e.g. limited intake of antioxidants due to restriction of certain fruits and vegetables) and 5) genetic factors and racial disparities (as reviewed by Kalantar-Zadeh & Balakrishnan [66]).

Dyslipidemia

A number of factors contribute to the accelerated atherosclerosis in ESRD population. One of them is dyslipidemia which in some studies reported the prevalence rate as high as 80% [67, 68]. Dysregulation of lipid metabolism in this population is characterized by hypertriglyceridemia, elevated very low-density lipoprotein (VLDL) and reduced plasma HDLC. Interestingly, plasma levels of total cholesterol and LDLC are usually within normal limits [15].

In normal metabolism, liver secretes TAG rich VLDL into the circulation. Lipoprotein lipase (LPL), an endothelial enzyme, converts TAG in VLDL into free fatty acid, allowing uptake and utilization by peripheral tissue. As VLDL loses its TAG content, it becomes

intermediate density lipoprotein (IDL) and eventually LDL that is rich in cholesterol. LDL delivers cholesterol to extrahepatic tissue for synthesis and maintenance of cell membrane. HDL on the other hand is involved in reverse-cholesterol transport system, by removing excess cholesterol from extrahepatic cells via several mechanisms. HDL acquires lipids by: 1) action of ATP binding cassette transporter A-1 (ABCA1) that mediate the transfer of phospholipids and some unesterified cholesterol; 2) lecithin cholesterol acyltransferase (LCAT) that converts free cholesterol on the surface of nascent HDL into cholesteryl ester before sequestering it into the core of HDL particle - this step is important to ensure maximum uptake of cholesterol from extrahepatic tissue and maturation of HDL particle. HDL can unload its cholesterol either through binding with SR-B1 for liver uptake/excretion in bile or by transferring it to VLDL/LDL in exchange of TAG, mediated by cholesteryl ester transfer protein (CETP) [63]. However, the indirect pathway (i.e. through CETP) is believed to be pro-atherogenic due to the transfer of cholesterol from HDL to the pro-atherogenic VLDL/IDL [63].

In ESRD patients, despite having normal or even subnormal plasma total cholesterol, they are at high risk of atherosclerotic vascular diseases [69]. This is partly contributed by a delayed TAG rich lipoprotein catabolism which is illustrated in **Figure 2-5.** ESRD induced deficiencies of LPL that is responsible for removal of two-third of fatty acids content on VLDL for uptake by extrahepatic tissues [70]. This lead to accumulation of ApoB-containing lipoproteins (TAG rich) which are highly atherogenic because they are readily oxidizable and taken up by macrophages leading to formation of foam cells in the artery walls [69].

Vaziri et al [18, 69] reviewed evidences to explain lower HDL particle and its cholesterol contents in ESRD. Based on these evidences, it is summarize that: 1) hepatic expression and production of ApoA1, which is the principal protein component of HDL particle is decreased; 2)

expression of acyl-CoA:cholesterol acyltransferase-1 (ACAT) is increased – this cause retention of cholesterol within the cells due to entrapment of free cholesterol from HDL uptake; 3) hepatic production and activity of LCAT is reduced; and 4) HDL uptake by the liver for removal and degradation occurs via HDL endocytic receptor (irreversible) instead of SRB1 (reversible). Several lines of evidence also indicate that activity of CETP is increased in this population [71]. CETP mediates the transfer of cholesterol ester from HDL to VLDL remnants in exchange for TAG. In summary, CKD/ESRD induces dyslipidemia which causes increased influx, impaired efflux leading to accumulation of lipids in the artery walls which is believed to accelerate atherosclerosis in this population.

Malnutrition, inflammation, oxidative stress and their relation to CVD

Malnutrition, inflammation and oxidative stress interlink has been recognized as the 'evil axis' for poor survival rates in dialysis patients [20]. This is based on the fact that up to two third of dialysis patients show evidence of protein energy malnutrition, chronic inflammation and increased oxidative stress. In fact, nutritional and inflammatory markers such as serum albumin, CRP and IL-6 have been identified as the strongest predictor for mortality and morbidity in this population [72, 73]. It is believed that there is overlap between malnutrition and inflammation in term of their mechanism and their relation to CVD in this population [74]. A brief discussion on each of these components follows:

Malnutrition

Malnutrition is highly prevalent in ESRD population. It has been reported that, malnutrition present in 18-70% of ESRD patients undergoing hemodialysis [75]. Various terms have been used to describe a syndrome of adverse changes in body composition, inadequate nutritional

intake, and loss of protein/energy stores in this population. These terms include malnutrition, protein-energy-malnutrition, malnutrition-inflammation-atherosclerosis syndrome, and uremic cachexia [75]. However, the International Society of Renal Nutrition and Metabolism (ISRNM) suggests the use of the term - protein energy wasting (PEW) - to describe concurrent losses in protein and energy stores and facilitate the diagnosis and identification of this condition [12].

Based on the consensus statement by ISRNM, the etiology of PEW in this population is multifactorial and not limited to insufficient dietary intake alone [12]. In fact, uremic milieu is speculated to be responsible for alteration in appetite-regulating hormone such as ghrelin, resulting in chronic anorexia [76]. Moreover, HD patients also experience 12 to 20% increase in resting energy expenditure related to HD procedure [77], or the presence of comorbidities. Hypercatabolism in this population is also linked to persistent inflammation. Inflammation contributes to PEW by several mechanisms: 1) increase resting energy expenditure that triggers starvation response even in well-fed individuals [12, 78]; 2) pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL) cause muscle protein breakdown [79] and act on the central nervous system to decrease appetite leading to anorexia [80]; and 3) the increase in positive acute phase protein (i.e. CRP) leads to decreases in visceral protein stores (e.g. by reducing synthesis and half-life of serum albumin) [81]. In addition, dialysis procedure itself is also catabolic in nature, contributing to PEW by causing nutrient losses, triggering inflammatory response and leading to loss of residual renal function [12]. Potential causes of PEW are listed in **Table 2-3**.

Inflammation

Inflammation can be defined as a complex body response to injury [82], destruction of tissue or harmful stimuli characterized by production of cytokines (e.g. interleukins and tumor necrosis factor) which in turn stimulate secretion of acute phase protein (C-reactive protein). The initial reaction in response to damaging stimuli is normal; however, persistent inflammation is harmful as it may result in organ and vascular damage.

In ESRD population, inflammation is relatively an old problem that has been reported since 1980s [6]. However, only recently has chronic inflammation been recognized as an established risk factor that plays a central role in various co-morbidities especially CVD, PEW and is an independent predictor for mortality [6]. Pathophysiology of chronic inflammation in ESRD patients is complex due to its inter-twined relation with PEW, however each contributes independently to mortality risk [11]. Common causes of chronic inflammation that are traditionally reported include foreign body exposure (e.g. arteriovenous graft and dialyser), depletion of antioxidant, oxidative stress, and pre-existing co-morbid diseases [74, 83]. Some other causes include intercurrent clinical events (e.g. periodontal diseases) and dialysis related causes (e.g. infection of dialysis access and volume overload) as presented in **Figure 2-6**.

Pro-inflammatory cytokines have been postulated to induce and mediate the lean body mass catabolism in dialysis patients, however their exact role is not completely understood. One of the mechanisms proposed is through impairment of insulin signaling activity (e.g. suppression of insulin receptor-1-associated phosphatidylinositol 3-kinase activity) which subsequently leads to stimulation of ubiquitin-proteasome proteolytic system and activation of caspase-3 [84, 85]. Similarly, pro-inflammatory cytokines also suppress the expression and function of insulin-like

growth factor-1, which is an anabolic growth factor [86]. Moreover, IL-1 and TNF-α also have been shown to cause anorexia by acting on central satiety center, leading to suppressed food intake [85]. Taken together, pronounced catabolic effects of pro-inflammatory cytokines on muscle catabolism is due to combined suppression of food intake and increased protein breakdown which subsequently leads to overall poor nutritional status [85].

Oxidative stress – the missing link

Oxidative stress (OS) is defined as a state of imbalance between oxidant production and their degradation by antioxidant system which leads to accumulation of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Over 90% of free radical production occurs during normal process of metabolism such as during metabolism of oxygen in mitochondria when oxygen that is being passing down the electron transport chain leaks away and turns into superoxide anion [87]. Additionally, ROS could be synthesized in phagocytic cells, at the vascular walls, and other tissues following exposure to a stimulus such as the presence of foreign particle or during inflammatory processes [14, 87]. When OS occurs, it results in oxidation of various macromolecules such as DNA, lipids and protein [11]. Oxidative modifications of lipoprotein particles especially LDL is thought to be an important step in the initiation of atherogenesis [63, 69].

In HD patients, OS has been recognized as an important factor that serves as a missing link between malnutrition and inflammation. OS is highly elevated in HD patients when compared to healthy-matched controls [88]. Impaired oxidative balance in HD patients is partly attributed to increased ROS production, reduced clearance, and poor antioxidant defense system [14]. Several lines of evidence support the notion that OS is linked to CVD based on the fact that oxidative modification of LDL, is the initial steps that triggers immune response. Oxidized LDL

instigates cascade of reactions including adhesion of circulating monocytes on endothelial cells, migration of monocytes into arterial intima and platelet activation, cytokines release and expression of tissue factor and by endothelial cells [14].

There are several factors that contribute to the impaired oxidative balance in HD patients. These include the underlying disease itself, HD procedures, poor dietary antioxidant intake related to malnutrition and persistent inflammation. Evidence indicates that residual uremic solutes such as $\beta 2m$, and homocysteine can become substrate for oxidative modification, thus potentiating their pathogenicity [11]. HD procedure on the other hand causes solute loss including enzymatic antioxidants. Furthermore, malnutrition condition, characterized by hypoalbuminemia also contribute to OS. This is because albumin molecules contain thiol groups that act as antioxidant in order to maintain plasma milieu in a reduced form. Therefore, diminished plasma albumin reduces overall plasma antioxidant capacity. Taken together, inflammation and malnutrition condition will have a synergistic effect on the risk of oxidative injury in this population [11].

Standard of care for nutrition in HD patients

Patients undergoing chronic HD require a comprehensive nutritional care given the fact that high prevalence of protein energy malnutrition (PEM) is largely contributed by inadequate nutritional intake [51]. The goal of the nutritional management for these patients is to maintain a good nutritional status through adequate energy, protein, vitamin and mineral intake while minimizing complications associated with uremia by maintaining blood chemistry within normal ranges [45]. It is also crucial to ensure patient's adherence on fluid restriction, potassium and sodium intake in order to control edema and electrolytes imbalance, given the limited capacity of HD to

completely remove fluid and minerals. Often, nutritional management also considers long term complications of HD such as bone mineral diseases (renal osteodystrophy) by monitoring phosphorus, calcium and vitamin D intake. All of these are achieved through carefully planned dietary regimens and periodic counseling from a renal dietitian.

Continuous monitoring of patient's nutritional status is crucial in order to achieve these goals. This process includes assessment of visceral protein status, body composition and nutritional intake. The widely used clinical indicator for assessment of visceral protein store in HD patients is serum albumin. This is due to the fact that it is readily available and has been identified as an independent predictor for hospitalization and mortality [85]. Beside that, serum transferrin and prealbumin levels are also frequently used in monitoring nutritional status. It is important to note that serum albumin and prealbumin level can fluctuate based upon hydration status, infection, inflammation and trauma while ferritin can be influenced by changes in iron stores or the presence of inflammation. Since albumin and prealbumin are negative acute-phase reactants, it is common to measure C-reactive protein (an acute phase reactant) when their levels are low. Beside biochemical profiles, simple anthropometric measurements such as weight and BMI along with diet records are also used to monitor changes in body composition and nutritional status over time. Furthermore, the use of composite indices comprised of diagnostic measures of body composition and dietary intake coupled with subjective assessment of overall well-being is also recommended [85]. One of the commonly used composite indices is Malnutrition-Inflammation Score (MIS). MIS is a quantitative scoring system that incorporates components relating nutritional status (BMI, serum albumin, serum total iron binding capacity, appetite and gastro-intestinal symptoms), functional status and comorbidity [89].

Standard dietary recommendation for HD patients include energy intake of 35 kcal/kg body weight (for adult patients <60 years old) and 30-35 kcal/kg body weight for older adults (>60 years old), while protein intake should be at least 1.2 g/kg body weight with 50% of the protein being of high biological value. This is the maneuver to prevent protein-energy malnutrition and to meet the increase in requirement due to the catabolic nature of the HD treatment. Moreover, controlling mineral intake particularly sodium (<2.0 g /day), calcium (2.0 g/day) and phosphorus (0.8-1.0 g/day) should also be emphasized in order to prevent acute complications and comorbidities. Standard of care for nutrition in HD patients is summarized in **Table 2-4**.

Strategies to reduce CVD

As discussed earlier, inflammation plays a central role in various comorbidities associated with ESRD including CVD. Carrero and Stenvinkel [6], have outlined treatment strategies in order to control inflammation and reduce the associated comorbidities (**Figure 2-6**). These multi-modal treatment approaches include medical, pharmacological, nutritional and life style interventions. First and foremost, comorbidities and potential dialysis related causes should be addressed and treated if possible. These include assessment and treatment for intercurrent illnesses such as periodontal disease and infectious complications of HD access.

When all of the medical issues have been addressed, it is recommended that various anti-inflammatory strategies in the forms of pharmacological, physical and nutritional intervention be considered. Some of the pharmaceutical products reported to have immunomodulating properties include statins, pentoxifylline (a nonspecific phosphodiesterase inhibitor with anti-inflammatory properties) and angiotensin-converting enzyme inhibitor. Furthermore, given that majority of dialysis patients are physically inactive, resistance training and aerobic exercise could also be

introduced as several studies indicate beneficial results on inflammation [82, 90, 91]. From the nutritional standpoints, there are several nutrients that merit further investigation based on evidence from animal and human studies. These nutrients include omega-3 fatty acids, vitamin E, and soy isoflavones and genistein. The following discussion will focusing on the two nutrients that form the basis of this dissertation project, namely, omega-3 fatty acids and vitamin E tocotrienols.

Potential nutritional agents to reduce co-morbid conditions:

Omega-3 fatty acids

Several different system of nomenclature exists for fatty acids. One of the commonly used is n-x system (also known as omega-x), which is based on the location of the first double bonds counted from the methyl therminus. Thus a fatty acid that comprised of two or more double bonds, one of which is located three or six carbon positions from methyl terminus is called omega-3 or omega-6, respectively (**Figure 2.3**). In general, omega-3 fatty acids can be elongated from α-linolenic acid (C18:3) (ALA) to the longer chain fatty acids [eicosapentanoic acid (EPA) (C20:5)]. EPA can be further elongated into docosahexanoic acid (DHA) (C22:6) in the peroxisomes via several complex steps. Because enzymatic conversion of ALA into EPA and DHA is limited in human, this necessitate reliance on dietary sources in order to maintain adequate levels of EPA and DHA [92].

Dietary sources and dietary intake recommendation

In the US, average dietary intake of omega-3 fatty acids is about 1.6 g/day or equivalent to 0.7% of total energy intake [93]. Major dietary source of omega-3 ALA include vegetable oils such as soybean, rapeseed, flaxseed and also walnuts. EPA and DHA are predominantly from cold-water

fish with varying concentration among species depending on environmental factors and whether the fish is farm-raised or wild. Some of the rich sources of EPA and DHA include sardines, Atlantic salmon, and pacific herring which provide more than 1g per serving size (3 oz.) [94].

Currently, there is no reference for normative range for blood and tissue concentration of omega-3 fatty acid. Nevertheless, American Heart Association has established evidence-based guidelines which recommends individuals with documented heart disease to consume about 1g/d of EHA+DPA [32, 92]. In the nephrology practice, there is no consensus renal dietary recommendation for omega-3 fatty acid intake, however the recent NKF KDOQI guidelines recommend for further study to identify the essential fatty acid status in CKD patients [95].

Absorption and transportation

Absorption and transportation of omega-3 fatty acids follow the same route as other long-chain fatty acids. Upon absorption by intestinal cells, they are resembled into TAG and packaged into chylomicrons, which are then transported into the thoracic duct via the lymphatic system and subsequently enter the blood stream for distribution to tissue and finally to be taken up by the liver.

Epidemiology and observational studies in general population

Earlier epidemiological studies from various population such as Greenlandic Eskimos [96] and Japanese coastal fishing village residents [97] indicate that higher fish intake were associated with lower risk for cardiovascular diseases. In the Nurses Health Study, fish intake and omega-3 fatty acids have been reported to have an inverse association with coronary heart disease (CHD). Women who consumed fish 1 to 3 times per month, once per week, 2 to 4 times per week and >5 times per week had lower risk of CHD death (21%, 29%, 31% and 34%, respectively) when

compared with women who rarely ate fish (less than once per month) [98]. Some epidemiological studies however failed to demonstrate any beneficial association of fish consumption and CHD / cardiac mortality including Health Professional Follow-up Study [99]and the US Physician Health Study [100]. It is speculated that the different in the outcome of these studies are due to differences in experimental design, dietary intake estimation, study population and variability in the end point studied [94]. It is however beyond the scope of this dissertation to discuss literature in the non-kidney disease population. Therefore, the following discussion is streamlined towards omega-3 research in dialysis population.

Omega-3 fatty acids status in dialysis patients

Recommendation for omega-3 fatty acids supplementation in dialysis patients is stemmed from the fact that omega-3 intake in dialysis population is much lower as compared to healthy control, partly due to monotony of their diet [32, 92] and non-palatable in taste because of uremia associated taste alteration [92, 101]. This is also supported by the fact that levels of omega-3 PUFA plasma and cell membranes of HD patients is significantly lower as compared to healthy subjects [31, 102]. Furthermore, HD patients who are fish eaters have been shown to have much lower mortality as compared to their counterpart [92]. In term of its cardiovascular effects, omega-3 has been shown to possess lipid altering properties (especially in reducing TAG), improved inflammatory markers and improve endothelial function related to its anti-thrombotic properties [103, 104].

Therapeutic effects of omega-3 fatty acids: lipid altering

The mechanisms involved for the observed effects of omega-3 fatty acids with regards to cardio vascular health is not clearly understood [94]. The potential mechanism by which omega-3

confers cardio-protective effects is summarized in **Table 2-5**. The most established effect of the omega-3 fatty acids is as a hypotriglyceridemic agent. Majority of the studies reviewed in this dissertation reported somewhat effects of omega-3 on lipid profiles with supraphysiologic omega-3 supplementation [29, 105, 106] (**Table 2-6**). In a longitudinal study by Schmitz and colleague, 4g daily of fish oil (80% of which containing omega-3 fatty acids) supplementation resulted in 53% reduction in TAG after 3 months of the supplementation [105]. In another study, Khajehdehi and colleagues reported that supplementation with 2g omega-3 per day in HD patients resulted in significant improvement in TC/HDLC, LDLC/HDLC and TAG/HDLC ratios [29]. Taken together, in hemodialysis patients, there is consistent evidence indicating the effectiveness of omega-3 supplementation on lipid profiles. This is also in concert with a comprehensive review of human studies showing that the supplementation of ~ 4g/d of omega-3 fatty acids from fish oil reduced TAG by 25 to 30 percent [104] .

Based on the mechanistic study, it is reported that omega-3 fatty acids can modulate two important component in lipid homeostasis, namely peroxisome proliferator-activated receptors (PPARs) and sterol regulatory binding protein (SREBP) [107]. This is partly explained due to omega-3's ability to inhibit SREBP conversion from its inactive to active form, affect cellular composition of the membrane, and bind to PPAR-α and –γ which promote β-oxidation and adipogenesis [107].

Therapeutic effects of omega-3 fatty acids: anti-inflammatory

Inflammation plays a central role in the accelerated atherosclerosis in HD patients. Friedman and Moe [92] reviewed several literatures available with regards to omega-3 fatty acids effects in HD patients, including on inflammatory markers – some of these studies are presented in **Table 2-6**.

In a case-control study involving 42 HD patients and 16 age-, gender-matched healthy control, supplementation with 2.4 g omega-3 fatty acids resulted in reduction of the inflammatory markers (TNFα and IL-6) [106]. Similarly, Saifullah and colleagues demonstrated that administration of omega-3 fatty acid with modest dose (1.3 g/day) resulted in 24% reduction in CRP, a surrogate marker for CVD [108]. In fact, reduction in CRP levels has been found to be independent of TAG reduction after 6 month of fish oil supplementation using 2 g fish oil daily (containing 1.6 g omega-3 fatty acids) [109]. However, in a systematic review of all fish oil/omega-3 fatty acid supplementation in human, it appears that omega-3 fatty acids is ineffective in reducing inflammatory markers [110]. This however has been criticized due to the inclusion of studies with relatively small number of subjects in healthy population [108], while it is generally accepted that the effect of supplementation would be more apparent in population with heightened inflammatory response such as in HD patients.

Mechanistic explanation of the anti-inflammatory effects of omega-3 fatty acids is illustrated in **Figure 2-8**. It is known that arachidonic acid (omega-6 fatty acid) can be converted into eicosanoid products (e.g. prostaglandins, leukotrienes, thromboxanes, lipoxins and epoxyeicosatrienoates) via cyclooxygenase, lipoxygenase, or cytochrome P450 arachidonic acid monooxygenase. Some of these arachidonic acid-derived eicosanoids promote platelet aggregation, vascular permeability, vasoconstriction and cytokines release; hence their pro-inflammatory and pro-thrombotic properties are implicated in the pathogenesis of CVD [92]. Because omega-3 fatty acids can incorporate into cell membrane phospholipids, this results in reduced content of arachidonic acid in the cells. As arachidonic acid is reduced, there is limited amount of substrate available for the synthesis of pro-inflammatory eicosanoids. In other words, omega-3 fatty acids exert their anti-inflammatory effects by competing with omega-6 fatty acids

for the incorporation into the cells and for the enzymes involved in the eicosanoids synthesis [92]. Moreover, in HD patients, there is a line of evident indicating that the metabolism of arachidonic acid is shifted from cyclooxygenase pathway to the lipooxygenase pathway, leading to producing a relatively more pro-inflammatory leukotrines [111]. Therefore, higher levels of omega-3 fatty acids particularly EPA and DHA could block the lipooxygenase pathway of eicosanoid production leading to less inflammatory eicosanoids [106, 111]. Reduced pro-inflammatory eicosanoids may alleviate inflammation by decreasing production of proinflammatory cytokines, and cell surface molecules such as intercellular cell adhesion molecules (ICAM) and vascular adhesion molecules (VCAM) that play a critical step in foam cell formation in the atherogenesis process [92, 112]. In summary, the molecular aspect of omega-3 fatty acids action on inflammatory response is well understood, however, this is yet to be translated into a positive outcome in clinical population.

Potential nutritional agents to reduce co-morbid conditions:

Vitamin E tocotrienol

Vitamin E is a group of compounds that possess a chromanol ring with a long chain attached to its 2-position. There are eight known naturally occurring vitamin E isomers namely α-, β-, δ-, γ-tocopherol (TT) and tocotrienols (TP) that differ in the number and position of methyl group in the chromanol ring (three methyl groups in α- isoforms; two methyl groups in β- and γ- isoforms; one methyl group in δ- isoforms). Vitamin E is a generic term used to describe all TT and TP isomers that qualitatively exhibit the biological activity of α-tocopherol. TT and TP exhibit similar chemical structures except the fact that TT possess a farnesyl (unsaturated chain) rather than a saturated isoprenoid C_{16} chain [113], characterized by the presence of three trans double

bonds (**Figure 2-4**). This explained the fact that TT has greater fluidity and superior cell accessibility thus confers a distinct biological activity than TP [34, 114].

Dietary Sources

Natural sources of vitamin E are lipid-rich plant products and vegetable oils. The plants accumulate vitamin E in oily seeds and fruits to prevent lipid peroxidation and protect from oxygen toxicity [113]. TP are widely available at high concentration in many type of fruits and vegetable (>0.5mg/100mg edible portions) while TT are limited to certain fruits and vegetable at a level that usually less than 0.1mg/100mg edible portion [115]. A good sources of TT can be found in cereal grains such as oat, barley and rye, while the richest natural sources of TT are found in annatto, palm and rice bran oil [33, 34, 116] (**Figure 2-10**). Given that, TP particularly γ-TP is the most abundant vitamin E in the US diet [117].

Absorption and transportation of vitamin E

Absorption mechanism of all vitamin E isomers are fundamentally similar as in other lipid molecules (reviewed in Rigotti 2007 [118]). Upon digestion, vitamin E is incorporated into mixed micelles and taken up by enterocytes. The uptake of vitamin E from the mixed micelles is hypothesized to occur via a simple passive diffusion as well as through intestinal scavenger receptor class B type I (SR-B1) [118]. However, the detailed cellular mechanism for intracellular trafficking of vitamin E is remained to be elucidated [118]. Once in the enterocytes, vitamin E is packed into chylomicron and secreted into the lymphatic system. Several studies indicate that there is no selective preferential between TP and TT forms – they are absorbed and secreted into chylomicrons in similar proportion of those occurring in the diet [118, 119]. Anwar and colleague [120] demonstrated that, an alternative pathways of intestinal vitamin E absorption

exist – this is occur through HDL-dependent mechanism in which vitamin E is directly secreted by intestinal epithelial cells into HDL. It is important to note that there is interindividual variability in intestinal absorption of vitamin E which range from 20 to 80%. This is partly due to intrinsic differences in expression and activity of SR-B1 and ABCA1, and influenced by the amount and quality of dietary fat in a meal [118, 121, 122]. Lipoproteins are the major transporter of lipid soluble antioxidants including vitamin E. Following a meal, chylomicrons deliver vitamin E to the liver parenchymal cells, while LDL and HDL transport most of vitamin E under fasting condition for hepatic uptake [118].

Regulation of vitamin E concentration

Biological half-life of TT is relatively shorter than TP (by 4.5 to 8.7 fold); α-, γ-, δ-TT have 2.3, 4.4 and 4.3 hours, respectively *vs* 20 hours for α-TP [33, 123]. This is explained by the fact that following hepatic uptake of vitamin E, there is selective preferential for α-TP to being re-secreted into the plasma due to differential affinity for various vitamin E forms in α-Tocopherol Transfer Protein in the following order: α-TP (100%) > α-TT (12%) > γ-TP (9%) > δ-TP (2%) [124, 125].

Since TP discovery some decades ago by Evans & Katherine Bishop, a vast majority of studies have been focusing on the therapeutic role of TP [33]. The other forms of vitamin E compound is remained poorly understood in part due to the abundance of α-TP in human plasma and lack of bioavailability of the other vitamin E isomers [113]. However, TT have recently gained increasing scientific attention as current research indicates that they possess a different therapeutic role than TP due to their eminent anti-oxidative, neuroprotective, anti-hypercholestrolemic and confers a distinct molecular target [33, 34, 113]. The following

discussion will be emphasizing on TT. Potential therapeutic effects of TT are summarized in **Table 2-7**. It should be noted that, a vast majority of the literature pertaining to the clinical intervention of vitamin E in HD population are in the form of TP supplementation – this is summarized in **Table 2-8**.

Therapeutic effects of tocotrienol: Antioxidant

Imbalance between the generation of reactive species and antioxidant defense system lead to oxidative stress and subsequently macromolecules damage including DNA, lipids and protein [11]. Oxidative stress has been shown to involve in the pathogenesis of various chronic diseases such as cancer and atherosclerosis. As reviewed earlier in this chapter, oxidized LDL play an important role in the development of atherosclerosis, by attracting accumulation and differentiation of monocytes, followed by massive deposition of cholesterol and formation of lipid-filled foam cells on the artery wall. The differentiated monocytes (i.e. macrophages) in turn express various cytokines which trigger the inflammatory response. Therefore, it is postulated that antioxidant including vitamin E tocotrienol can prevent DNA damage and lipid peroxidation by neutralizing free radical and halt further propagation of atherosclerosis [34].

It is well established that, TT, as TP, exhibit antioxidant activity due to phenolic group in the chromane ring which neutralizes peroxyl radicals by conversion to relatively stable phenoxyl radicals [126, 127]. These less reactive radicals can be reduced back to its original form by vitamin C or other thiol antioxidants [116]. However, Yu et al [128] reported that polyunsaturated phytol side chain of TT also exhibit antioxidative activity. Furthermore, TT also has a higher recycling efficiency from chromanoxyl radicals due to its position closer to the membrane surface area as demonstrated in the study using nuclear magnetic resonance

spectroscopy [129]. This is consistent with other reported publication that TT have a superior antioxidant activity when compared to TP [36, 129, 130]. For example, *in vitro* experiment demonstrated that that α-TT is 40 times more effective against lipid peroxidation in rat liver microsomal membranes and is 6.5 times more efficient in protecting cytochrome P-450 from oxidative damage when compared to α-TP [34, 36]. *In vivo*, TT has been shown to be at least as equally effective as TP considering physiological processes (e.g. absorption, distribution, metabolism and excretion) that may limit its antioxidative properties [116]. Recent study using type-2 diabetic rats supplemented with tocotrienol-rich fractions (TRF) showed that the activity of antioxidant enzymes (i.e. superoxide dismutase, glutathione peroxidase) were restored and malondiadehyde (MDA), an oxidative stress marker, were reduced when compared to the untreated group. The antioxidant activity of TT has been postulated to confer protection against diabetic nephropathy in these rats [131].

In human, data on the antioxidative role of tocotrienols in chronic diseases is scarce. In a study by Chin and colleagues, supplementation with 160 mg of TRF for 6 months in healthy adults failed to improve erythrocyte antioxidant enzymes (i.e. superoxide dismutase and glutathione peroxidase) and MDA levels [132]. In summary, despite a clear anti-oxidative effect of TT has been shown in cell and animal studies, this has yet to be demonstrated in clinical population.

Therapeutic effects of tocotrienol: Anti-inflammatory properties
As discussed earlier, inflammatory mediator such as cytokines are implicated in the development of atherosclerosis in part by promoting expression of cell adhesion molecules and generation of free radicals [11, 64]. Therefore, dietary compound possessing anti-inflammatory properties such as TT has received a greater scrutiny recently. TT has been shown to reduce arachidonic acid, a

primary substrate for the synthesis of pro-inflammatory eicosanoids, in hereditary hypercholestrolemic swine [133]. In a recent systematic review by Frank and colleagues, they illustrated that TT could alleviate pro-inflammatory eicosanoids from arachidonic acid based on two pathway; 1) through inhibition of glutame-induced production of phospholipase A$_2$, which is the enzyme that catalyzes production of arachidonic acid from the cell membrane phospholipids, and 2) through inhibition of cyclooxygenase-2 and c-Src kinase resulting in reduced production of pro-inflammatory eicosanoids (Figure 2-11) [116]. This is also consistent with the lowering of the 'end-point' of the eicosanoid products such as TNFα, and interleukins in lipopolysacharides-induced human monocytic cells following incubation with TRF [37]. Moreover, several lines of evidence suggest that TT could block activation of NFκB [37, 134], a family of transcription factors that plays a central role in regulation of genes critical for inflammation and immunity. Interestingly, such effects were not seen with TP [134].

In hemodialysis population however, there is no study on TT as yet. Nevertheless, several studies in general population indicate a positive effect on inflammatory markers. In a study by Heng et al [135], subjects were recruited based on two different age group (young adults with average age of 32 and old adults with average age of 52), and TRF was administered for 6 months. They found that TRF supplementation in older adults resulted in lower CRP levels, which has been regarded as a prognostic indicator for cardiovascular events and mortality in ESRD patients [6, 7].

Therapeutic effects of tocotrienol: Lipid altering properties

TTs have been consistently demonstrated in the literature to possess lipid altering effects especially in cell and animal studies. In a study using chicken, TC and LDL-C levels were

reduced by 22% and 52%, while HDLC-LDLC ratios were improved by 123-150% in the animal that were fed a small doses of tocotrienol-rich fraction for four weeks [136]. Furthermore, the same authors also indicate that each of the TT isomer has variable impact on the serum lipids, with γ- and δ-TT considered as the most potent. In the experimentally induced hyperlipidemic rats, supplementation with a range of TRF concentration resulted in decreased in lipid parameters (TAG, LDLC) in dose-dependent manner with the optimum dose identified at 8mg TRF/kg/day [137].

It is generally accepted that the hypocholestrolemic effect of TRF can be explained by suppression of mevalonate pathway that serves as the basis for cholesterol biosynthesis. Sterol-inhibitory activity of TT is contributed by the side-chain unsaturation with ability to increase cellular farnesol which signals the proteolytic degradation of HMG-CoA reductase (HMGR), a rate-limiting enzyme of the cholesterol biosynthetic pathway [34, 138]. Therefore this lipid altering properties is unique for TT but not TP. Furthermore, in cell study, Song & Debose-Boyd [139] mechanistically explained that δ-TT suppressed cholesterol biosynthesis by stimulating ubiquitination and degradation of HMGR, and blocks processing of sterol regulatory element-binding protein (SREBPs), the transcription factors that involved in synthesis of enzyme involved in sterol biosynthesis. They also showed that TP neither accelerate HMGR degradation nor block SREBPs processing, in concert with the notion that lipid-altering effect is the unique properties of TT. Another mechanistic study from HepG2 liver cell line, hycholesterolemia induced-mice and borderline hypercholestrolemic patients summarized that the lipid lowering effect of TT, particular γ- and δ- are due to: 1) suppression of the upstream regulators of lipid homeostasis genes (i.e. ApoB100, SREBP, HMGCR and diaclyglycerol O-acyltransferase 2)

resulting in reduced TAG, cholesterol and VLDL biosynthesis; and 2) enhancement of LDL efflux via induction of LDL receptor expression [140].

However, the effects of TT particularly in lipids in human are rather ambiguous. In a double-blind, cross-over clinical study, Qureshi and colleagues demonstrated that supplementation with TRF in hypercholestrolemic human subject resulted in 31% decreased in serum cholesterol during 4 week period when they were given 200mg γ-TT/day as compared to the time when the same subjects were given 300mg corn oil/day [141]. Furthermore, the same authors demonstrated that TTs supplementation in patients who took statins reduced statins-related side effects [142]. On the other hand, Mensink et al [143] provided TRF supplementation (140mg TT, 80mg TP per day) to 20 men for 42 days showed no improvement in lipoprotein profiles despite changes in plasma TP and TT concentration. Similarly, administration of 200mg TT/day for 28 days in healthy hypercholestrolemic volunteers failed to show any effects on blood lipids [144]. In a dose escalation study involving 32 normal healthy men, supplementation with variable doses of TRF ranged from 80mg/day to 320mg/day also did not showed any changes in lipid profiles [145].   Taken together, the lack of consistency in human results may be due to: 1) variability in absorption of TT (e.g. with meal/ without meal); 2) different in doses; and 3) different clinical population [35]. **Table 2-9** summarized studies reporting the global effects of TT supplementation (lipids, inflammation and anti-oxidative effects) in healthy volunteer. As of now, there is no study reporting the effects of TT supplementation in HD patients.

Applying metabolomics for nutrition research in ESRD population

Metabolomics definition

'Omics' is a general term referring to interdisciplinary system biology to denote studies on a large-genome wide scale including genomics, transcriptomics, proteomics, and metabolomics. The 'omics' science has emerged since several decades ago, much attention has been focused on sequencing the human genome and annotating the human proteome; however, the current focus is on its application in finding a novel insight to understand the processes in cellular metabolism in relation to health and diseases [146, 147].

Metabolomics is the latest of the omic sciences that utilized cutting edge analytical instruments to quantitatively measures metabolites and detect their changes in biological fluids and tissues in conjunction with pattern recognition approach and bioinformatics [147]. In other words, metabolomics approach in human scrutinize the human metabolome – that is the complete collection of human metabolites comprising of a diverse small molecules chemical entities such as peptides, amino acids, nucleic acids, carbohydrates, vitamins, drugs and organic acids that are part of biochemical and cellular processes or can be ingested by humans [146]. Because of a diverse role of metabolites in serving as building block of various macromolecules, fueling all cellular processes, and acting as messengers and buffers in intracellular/extracellular events following environmental insults, they together with genome and proteome define what and who we are [146].

However, in contrast to the genome and proteome, metabolome is not well defined due to the fact that it does not dictate by the genome alone because it can be affected by various factors such as environmental insults (exogenous factor) or even the indigenous microflora in the gut

(endogenous factor) [146, 148]. For example, a disease process altered biochemical imbalance in the body that is transpires in peculiar metabolites that can be measured in biological fluids and tissues. On the other hand, organism's genome is constant while the change in transcriptome and proteome does not always translate as phenotypic alteration. Given that, compared to the other omics platform, metabolomics has a greater potential to impact clinical health practices due to the fact that metabolites represent integrative information of the phenotype in response to genetic and environmental changes [149]. Taken together, a comparative analysis of metabolite profile in disease *vs* non-disease states enable disease etiology, stages of progression and diagnostic biomarkes to be ascertained [149], exemplifies its application in clinical medicine for prognostic or predictive interpretation of the disease status. It is important to note that despite a number of advantages offer by metabolomics over conventional analyses, our knowledge on the composition of the metabolome is currently incomplete [150], thus may limit overall application in understanding the disease pathophysiology, diagnosis and treatment.

Metabolomics methodology

Metabolomics comprised of two generalized experimental strategies namely: 1) non-targeted metabolic profiling and 2) targeted metabolic profiling [150]. Non-targeted metabolites profiling is referring to a holistic study, an unbiased approach of data analysis without prior knowledge about the data set/ the represented metabolites. This approach usually leads to discovery of new ideas on the disease process, hypothesis-generating and a potential new ways of diagnosis [146, 150].

However, potential issues arise with the non-targeted metabolite profiling / pattern recognition is the fact that metabolic profile may not be specific to a disease condition while

confounders may not be accounted for, leading to problems in understanding the overall disease process [151, 152]. Therefore, a second strategy, a targeted metabolite profiling is often used to focus on quantitative changes of metabolite of interest with priori knowledge pertaining to their involvement in the disease process. This closed profiling approach is also being used to validate a biomarker after it is being discovered [152].

One of the common multivariate data analysis uses for metabolomics is a principle component analysis (PCA). In PCA, a new smaller set of uncorrelated variables, known as principle component (PC) is formed from a large set of related variables (e.g. NMR spectra from the plasma of HD patients), which represent the largest variation from the original data set. Using a multidimensional space, each of this PC is compared on a score plot, in which one score representing one observation (i.e. NMR spectrum from the respective individual). The contribution of each variable to the PC can also be calculated and visualized as a loading plot. Loading plot indicates variables (i.e. a part of the NMR spectrum) that responsible for the clustering and separation that correspond to the score plot [153]. Other statistical algorithm such as partial least square (PLS) is also commonly used when analyzing human metabolome. Unlike PCA, PLS is a supervised method of analyses which is an extension of PCA in which Y variables are added to connect with the information provided by X variables. Y variables represent additional information of the data set such as different group of intervention (intervention *vs* placebo) or different time of measurement (e.g. baseline *vs* end of study), biochemistry or clinical data [154]. In other words, PLS method helps to identify correlation between the measured bioassay (y-axis) with NMR spectra data (x-axis). PLS-discriminant analysis (PLS-DA), another method that commonly used, is a modification of PLS. PLS-DA is focused on maximum separation (discrimination) rather than maximum variation [155].

Metabolites profiling by nuclear magnetic resonance spectroscopy

Analytical tools that are available for metabolomics study are nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) such as liquid chromatography-MS (LC-MS) and gas chromatography-MS (GC-MS). GC-MS and LC-MS are robust and mature technology offering superb sensitivity in detecting metabolome [146]. However, both of the latter techniques have some drawbacks including relatively slower process, requires sample preparation/ separation (GC-MS), sample not recoverable (both LC-MS and GC-MS) and may suffer from alteration due to sample collection and handling, hence requiring stringent quality assurance [152]. On the other hand, NMR is more of a favorite tool for metabolomics due to its ability to provide a high resolution spectra, requires relatively less sample preparation hence more rapid analysis and more importantly non-destructive to the sample [146]. NMR techniques is based on the properties of the magnetic spin of the nuclei to determine chemical structures and concentration of a compound, thus nuclei having odd spin number such as ^{1}H, ^{13}C, ^{31}P and ^{15}N are commonly used. However, it is important to note that, the number of metabolites detectable using NMR techniques is relatively smaller when compared to LC-MS or GC-MS partly due to dependency on the magnetic strength to detect the chemical shift for metabolite identification, throughput and sensitivity [152].

Metabolomics application

Metabolomic approach has been used in various field including cancer, diabetes mellitus, and cardiovascular diseases. In cardiovascular research, metabolomics is used predominantly in elucidating etiology and identifying various biomarkers in the diseases pathophysiology [152]. Sebatine and colleagues demonstrated that a non-targeted approach of metabolomics were able to

differentiate the metabolomics profiles of those who had clear-cut inducible ischemia than those who did not (all patients underwent stress test for evaluation of possible myocardial ischemia) [156]. This has allowed them to analyzed metabolic disturbance before and after stress testing to identify potential novel biomarkers of coronary ischemia. Patients with clear-cut inducible ischemia showed abnormality in the levels of gamma aminobutyric acid, uric acid, citric acid and some other unidentified metabolites [156].

As far as ESRD patients is concerned, there are very limited studies that utilized the metabolomics approach to study the disease process or potential biomarkers identification to help in diagnosis and treatment. Recently, Ando and colleagues conduct an explorative study to assess metabolic response in ESRD patients undergoing chronic HD in a time course manner - before, during and after the HD treatment using plasma and dialysate sample using ^{1}H NMR spectroscopy [157]. They found a good correlation of creatinine in the plasma and dialysate which indicating that dialysate solution could be used as a non-invasive measurement of metabolic profile in HD patients in the future. Interestingly, they also found that additional metabolites were formed during HD procedure [157], suggesting the catabolic effect of the HD treatment.

TABLE 2-1. Overview of kidney functions

Function	Descriptions
Waste elimination	• Removal of metabolic waste products (urea, creatinine, uric acid) • Elimination and detoxification of drugs and toxins
Fluid balance	• Involve in removal and reabsorption of water to maintain fluid balance (via action of antidiuretic hormone, atrial natriuretic peptide, aldosterone)
Acid-base regulation	• Involve in secretion, excretion and reabsorption of H^+, HCO_3^-, NH_4^+, PO_4^{++} to maintain blood pH
Electrolytes balance	• Involve in excretion and reabsorption of electrolytes such as sodium, potassium, chloride, and bicarbonate to maintain homeostasis.
Mineral metabolism	• Control of mineral metabolism through endocrine synthesis (1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol) and excretion of phosphorus.
Endocrine functions	• Regulation of systemic blood pressure (renin, angiotensin, prostaglandin, nitric oxide, sodium homeostasis) • Production of erythropoietin
Metabolic process	• Regulation of metabolic processes (gluconeogenesis, lipid metabolism) • Degradation and catabolism of peptide hormones (insulin, glucagon, parathyroid hormone) and low molecular weight protein (β_2-microglobulin and light chain)

(Adapted from Himmelfarb et al 2010 [46])

TABLE 2-2. Current CKD staging based on GFR used by KDIGO

GFR Category	GFR (ml/min/1.73 m2)	Terms
G1	≥ 90	Normal or high
G2	60-89	Mildly decreased*
G3a	45-59	Mildly to moderate
G3b	30-44	Moderately to severe
G4	15-29	Severely decreased
G5	<15	Kidney failure

(Source: KDIGO, 2012 [49])

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate
* Relative to young adult level
In the absence of evidence kidney damage, neither GFR category G1 nor G2 fulfill the criteria for CKD

TABLE 2-3. Potential causes of protein energy wasting in CKD/ESRD population.

Causes	Notes
1. Decreased protein and energy intake	a.Anorexia - Dysregulation in circulating appetite mediators - Hypothalamic amino acid sensing - Nitrogen-based uremic toxins b.Dietary restrictions c.Alterations in organs involved in nutrient intake d.Depression e.Inability to obtain or prepare food
2. Hypermetabolism	a.Increased energy expenditure - Inflammation - Increased circulating proinflammatory cytokines - Insulin resistance secondary to obesity - Altered adiponectin and resistin metabolism b.Hormonal disorders - Insulin resistance - Increased glucocorticoid activity
3. Metabolic acidosis	
4. Decreased physical activity	
5. Deceased anabolism	a.Decreased nutrient intake b.Resistance to GH/IGF-1 c.Testosterone deficiency d.Low thyroid hormone levels
6. Comorbidities and lifestyle	Comorbidities (diabetes mellitus, CHF, depression, coronary artery disease, peripheral vascular disease)
7. Dialysis	a.Nutrient losses into dialysate b.Dialysis-related inflammation c.Dialysis-related hypermetabolism d.Loss of residual renal function

(Source: Carrero et al 2013 [12])

TABLE 2-4. Dietary recommendations for patients undergoing chronic hemodialysis.

Nutrients	Recommended Intake
1. Calories (kcal/kg body weight)	30 – 35*
2. Protein (g/kg body weight)	1.2-1.4
Protein (% of total calories)	15-25
3. Carbohydrate (% of total calories)	50-60[#]
4. Fat (% total calories)	25-35
5. Cholesterol (mg/day)	<200
6. Saturated fat (% of total calories)	<7
7. Sodium (g/day)	<2.0
8. Calcium (g/day)	2.0
9. Phosphorus (g/day)	0.8 – 1.0

(Adapted from: Ikizler 2010 [85])

Note: *35 kcal/kg body weight/day if <60 years old and 30-35 kcal/kg body weight/day if >60 years old.
Carbohydrate intake should be provided at a reduced amount for patients with hypertriglyceridemia.

TABLE 2-5. Potential mechanism of actions of omega-3 fatty acids on cardiovascular disease

Omega-3 fatty acids properties	Remarks
1. Hypotriglyceridemic	• Reduce fasting and postprandial TAG
2. Anti-inflammatory	• ↓ inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8)
	• ↓ generation of reactive oxygen species
	• ↓ arachidonic acid derived eicosanoids
3. Antithrombogenic	• ↓ paletelet agregation
4. Retard growth of the atherosclerotic plaques	• ↓ adhesion molecules expression (e.g. VCAM-1, ICAM-1)
5. Anti-arrhythmias	• Prevent calcium overload
	• Inhibit voltage-gated sodium channel
6. Mildly hypotensive	• Unknown mechanism

(Adapted from Connor 2000 [158]; Kris-Etherthon et al 2002 [94])

Abbreviations: TAG, triacylglycerol; TNF- α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1.

Note: This figure shows potential mechanism of omega-3 fatty acids in reducing risk for cardiovascular diseases. This is contributed by hypotriglyuceridemic, anti-inflammatory, antithrombotic, antiarrythmias and anti-atherosclerotic properties of omega-3.

TABLE 2-6. Selected studies reporting the effects of omega-3 supplementation in HD patients

Study	Design	Groups	Dose	Therapy Duration	Outcome Measures
Khajehdehi et al [29]	Randomized, placebo-controlled trial	4 different treatment groups (n=60) 1. Fish oil 2. Corn oil 3. Sesame oil 4. Placebo	1.5 g omega-3 /day	8 weeks	Ratios of HDLC/LDLC, TC/HDLC and TAG/HDLC decreased significantly in fish oil and corn oil treated groups.
Kalantar-Zadeh et al [159]	Concurrent control	Hypoalbuminemic HD patients: 1. intervention (n=20) 2. control (n=20)	0.46 g EPA	4 weeks	↑ in albumin in intervention group Non-significant reduction in CRP
Perunicic-Pekovic et al [106]	Case-series pre-post test	1. Age, sex matched control (n=16) 2. HD patients (n=42)	2.4 g omega-3 /day	8 weeks	↓ in IL-6, TNF-α ↑ in HDLC & albumin
Schmitz et al [105]	Randomized, placebo-controlled	1. Omega-3 (n=12) 2. Placebo (n=12)	4 g omega-3 /day	12 months	↓ in TAG by 53% Lower incidence of thrombosis in the treatment group (17% vs 75%)
Saifullah et al [108]	Randomized, placebo-controlled	1.Omega-3 (n=18) 2.Placebo (n=9)	1.3 g omega-3 /day	12 weeks	No significance changes in TAG Decreased in CRP
Tayyebi-Khosroshahi et al [160]	Non-randomized, placebo-controlled	1.omega-3 (n=37), 2.control (n=38)	3 g omega-3 /day	8 weeks	MDA significantly reduced in omega-3 group. No changes in antioxidant enzymes (SOD, FRAP)
Poulia et al[161]	Single blinded, randomized, cross-over trial	HD patients (n=25)	0.9g EPA + 0.8g DHA + 8mg α-TP, OR α-TP alone	4 weeks on each treatment, 2 weeks washout period.	No effects on serum lipids and CRP.

Abbreviations: EPA, eicosapenthanoic acids; DHA, decosahexanoic acids; TP, tocopherols, TAG, triacylglycerols; CRP, C-reactive protein; IL-6, interleukin-6; TNF-α; tumor necrosis factor alpha; MDA, malondialdehyde; SOD, superoxide dismutase; FRAP, ferric reducing ability of plasma.

TABLE 2-7. Potential therapeutic effects of tocotrienols.

Tocotrienols properties	Remarks
1. Antioxidant	• TTs are more potent radical scavenger and has greater antioxidant activity again lipid peroxidation in liposome than TPs. • TT is more effective in the protection of cytochrome P450 against oxidative damage when compared to TP. • This is due to faster cellular uptake; faster recycling from the respective chromanoxyl radical forms in liposomal membrane and lipoproteins; higher inter-membrane mobility.
2. Anti-inflammatory	• ↓ inflammatory cytokines (CRP, TNF-α, IL-4, IL-6, IL-8, NFκB) • ↓ generation of reactive oxygen species • ↓ arachidonic acid derived eicosanoids
3. Lipid altering effects	• Hypocholestrolemic effects is unique to TT not TP • This is due to post-transcriptional suppression of HMG-CoA reductase protein; enhance ubiquitination of HMG-CoA reductase.
4. Effects on suppression, regression and progression of atherosclerosis	• Suppression of atherosclerosis is related to reduction in oxidative stress, total cholesterol, increased in HDL-C; ↓ adhesion molecules expression (e.g. VCAM-1, ICAM-1) • Do not regress atherosclerosis in experimental animals • Conflicting results in progression of atherosclerosis.
5. Antithrombogenesis	• δ-TT is a potent inhibitor of platelet aggregation. • TT reduced serum levels of thromboxane-B2 and platelet factor 4

(Reviewed in Frank et al 2012 [116], Prasad 2011 [35] and Vasanthi et al 2012 [34])

Abbreviations: TT, tocotrienol; TP, tocopherols; CRP, C-reactive protein; IL, interlukin; TNF- α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; NFκB, nuclear factor kappa B; HDLC, high density lipoprotein cholesterol.

Note: This figure shows potential mechanism of tocotrienols in reducing risk for cardiovascular diseases. This is contributed by antioxidant, anti-inflammatory, lipid altering, anti-atherosclerotic and anti-thromogenic properties of tocotrienols.

TABLE 2-8. Selected studies reporting the effects of oral vitamin E (α-tocopherol) supplementation in HD patients

Study	Design	Groups (n)	Dose	Therapy Duration	Outcome Measures
Boaz et al.[25]	Randomized, double-blind placebo-controlled	1. HD pts α-TP (97) 2. HD pts placebo (99)	800IU/day	519 days (median)	No oxidative stress measures ↓ Cardiovascular end points (sudden death; myocardial infarction)
Bayes et al.[162]	Open label, untreated healthy control	1. HD pts (16) 2. Healthy control untreated (30)	400mg α-TP / dialysis session	3 months	↓ plasma MDA ↓ autoantibodies to oxLDL
Galli et al.[163]	Open label, untreated healthy control	1. HD pts (19) 2. Healthy control untreated (30)	800mg α-tocopheryl/d	3 weeks	↓ plasma TBARS ↑ nitric oxide ↑ plasma TP ↑ plasma GSH
Giray et al[164]	Open label, untreated healthy control	1. HD pts (36) 2. Healthy control untreated (36)	600mg α-TP/d	14 weeks	↓ plasma TBARS ↑ plasma GPX and SOD activity
Badiou et al.[165]	Open label, treated healthy control	1. HD pts (14) 2. Healthy control treated (6)	500mg α-TP/d	6 months	↓ plasma TBARS ↓ copper induced oxidation ↑ plasma lipoprotein α-TP
Diepeveen et al.[166]	Randomized, double-blind, placebo-controlled (factorial design)	1. HD pts given artovastatin + placebo α-TP (13) 2. HD pts given α-TP + placebo artovastatin (10) 3. HD pts given artovastatin + α-TP (11) 4. HD pts given placebo artovastatin + placebo α-TP (10)	800IU (536mg) α-TP/day	12 weeks	↓ in vitro LDL oxidazibility No effect on plasma oxLDL levels ↑ serum α-TP
Uzum et al.[167]	Randomized, placebo-controlled, with treated PD pts, untreated healthy control	1. HD pts α-TP 2. HD pts placebo 3. Healthy control untreated	300mg/day	20 weeks	↓ erythrocytes osmotic fragility levels in HD & PD ↓ MDA in HD ↑ serum α-TP

(Adapted from: Coombes & Fassett 2012 [24])

Note: This table shows a list of vitamin E intervention (Tocopherols) studies and their outcome measures in hemodialysis patients.

TABLE 2-9. Selected studies reporting the effects of tocotrienols in healthy volunteer and other clinical population

Study	Design	Groups	Dose	Therapy Duration	Outcome Measures
Radhakrishnan et al. [168]	Randomized, placebo-controlled trial	1. Healthy volunteer – TRF (n=16) 2. Healthy colunteer α-TP (n=15) 3. Control – placebo (n=17)	200mg of either TRF or α-TP	8 weeks	No different in immune cells modulation (i.e.T-helper, NK cells, B-lymphocytes, CD4:CD8 ratio)
Rasool et al. [145]	Randomized, blinded-end point, placebo-controlled trial	Healthy volunteer – 4 groups of dosage (n=36) 1. Placebo 2. 80mg/d 3. 160mg/d 4. 320mg/d	Various dosage: 80mg/d, 160mg/d, 320mg/d	8 weeks	No changes in TAS, ASBP, TC or LDLC. Group 160mg/d and 320mg/d showed significant improvement ASBP
Patel et al. [169]	Open label	Healthy volunteer (n=80)	400mg α-TP 400mg TT	20 weeks (mean) 1-96 weeks (range)	TT are transported into vital organs
Chin et al. [132]	Randomized, placebo-controlled trial	1. Healthy volunteer – placebo (n=30) 2. Healthy volunteer TRF (n=32)	160 mg TRF	6 months	Erythrocytes antioxidant enzymes (SOD, GluProx) Lipid profiles- significant improvement in HDLC in older adult group.

Abbreviations: TRF, tocotrienol-rich fractions; TP, tocopherols; TAS, total antioxidant status; TC, total cholesterol; LDLC, low density lipoprotein cholesterol, ASBP, aortic systolic blood pressure; SOD, superoxide dismutase; GluProx, glutathione peroxidase.

Note: This table shows a list of vitamin E tocotrienol intervention studies and their outcome measures in healthy volunteer and other clinical population.

FIGURE 2-1. International comparisons for incidents of end-stage kidney disease.
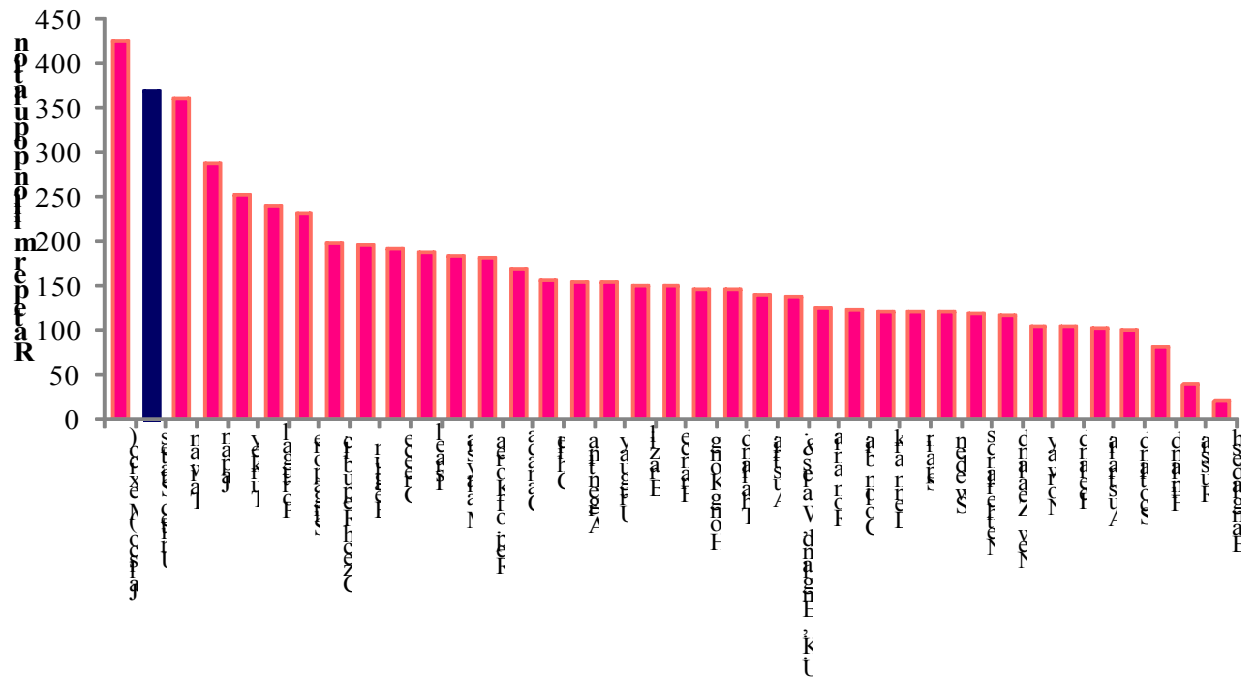
(Source: USRDS, 2012 [1])

Note: This figure shows incident rates of the reported ESRD cases worldwides in 2011. Mexiro reported the highest incident rate followed by the United States, Taiwan and Japan. Rates less than 100 per million population were reported in Scotland, Finland, Russia and Bangladesh.

FIGURE 2-2. Different types of treatment modality option for patients with end stage kidney disease.
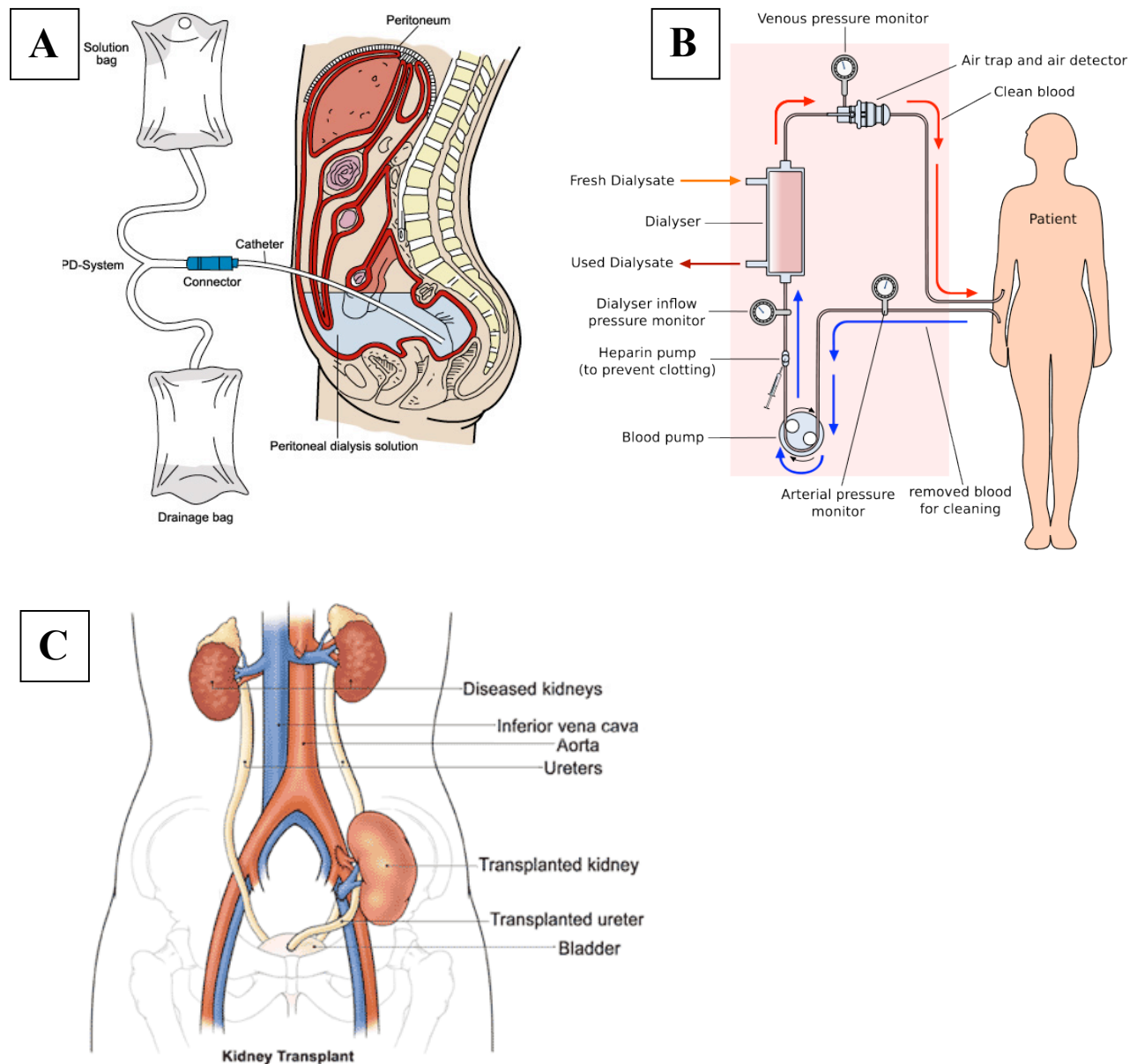
(Source: NKUDIC, 2013; Kidney Health Australia, 2013)

Legend: A- Peritoneal dialysis, B-Hemodialysis, C-Kidney transplantation

Note: (A) Peritoneal dialysis use patient's own peritoneum membrane to filter out fluids and other dissolved substances. (B) In hemodialysis modality, patient's blood is pump to pass through a dialyser membrane allowing diffusion of fluid and other waste substance across a semipermeable membrane. This dialysis modality is the most common in the US. (C) A new kidney from a donor is transplanted in iliac fossa, in the recipient's contra lateral side.

FIGURE 2-3. Schematic diagram of the increased cardiovascular burden in ESRD.
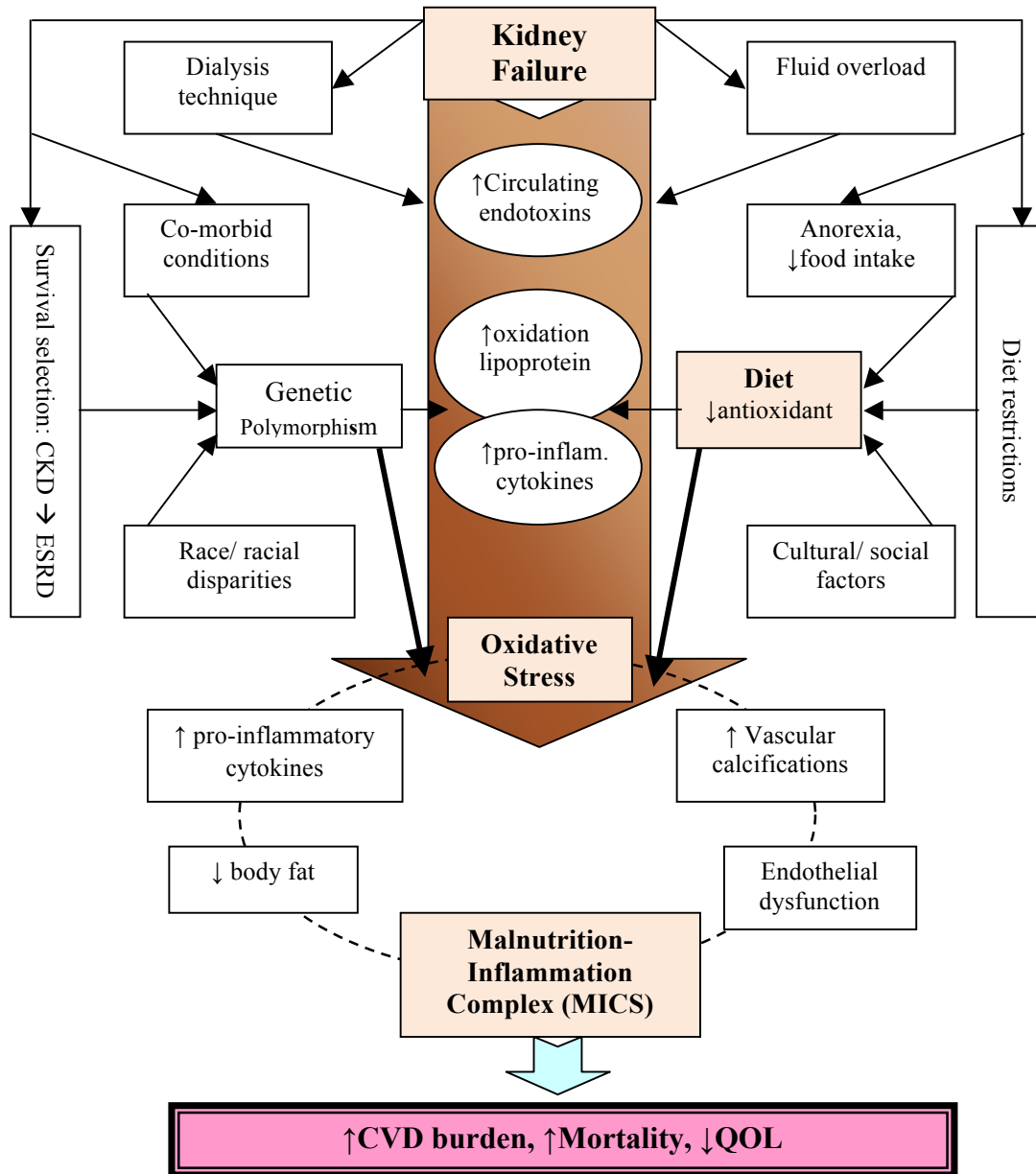
(Adapted from: Kalantar-Zadeh & Balakrishnan 2006 [66])

Abbreviations: CKD, chronic kidney disease; ESRD, end-stage renal disease; QOL, quality of life.

Note: This figure shows a proposed pathophysiology of cardiovascular disease in ESRD patients.

FIGURE 2-4. Schematic view of the atheroma's development
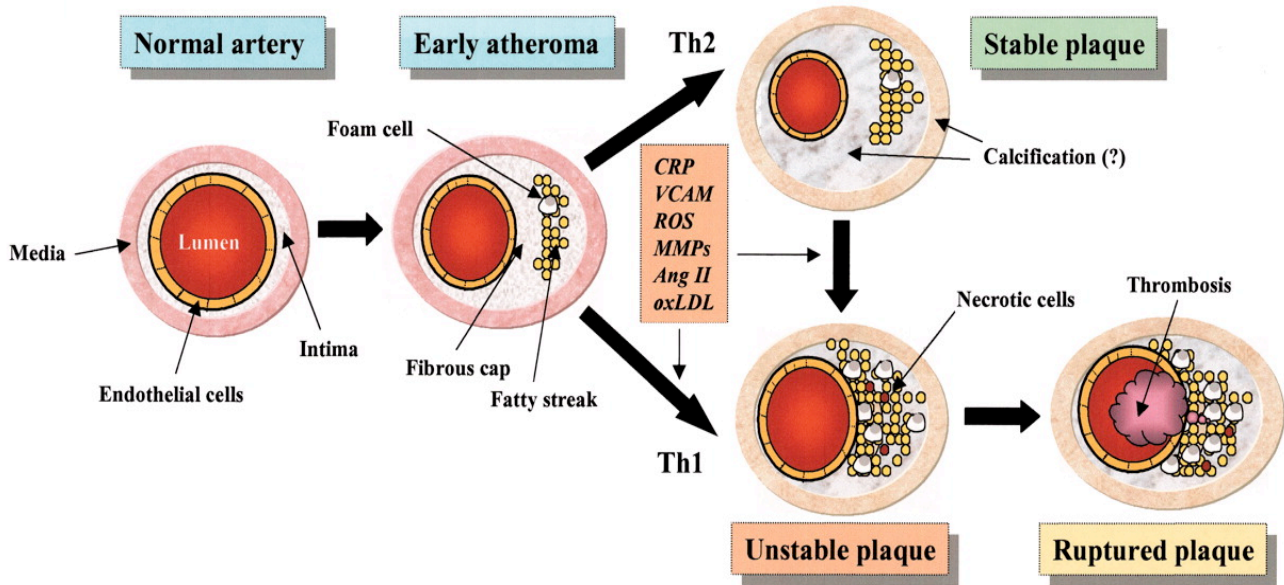
(Source: Stenvinkel et al 2003 [64])

Abbreviations: Th, T-helper cell; CRP, C-reactive protein; VCAM, vascular cell adhesion molecules; ROS, reactive oxygen species; MMPs, matrix metalloproteinases; AngII, angiotensin II; oxLDL, oxidized low-density lipoprotein.

Note: This figure shows atheroma's development processes. Normal human artery is comprised of three layers of cells – endothelial, the intima layer and the underlying tunica media. During early artherogenesis, fatty streak is developed as a result of recruitment of inflammatory cells and foam cells (from accumulated lipids). When dyslipidemia and inflammatory conditions persist, lipid pool grows. Activated leukocytes secrete proteinase which caused degradation of the extracellular matrix and ultimately cells death. Meanwhile, Th1 activity is stimulated by the present of inflammatory cytokines that limit the synthesis of new collagen which may thin fibrous cap which make it susceptible to rapture. Accute chronory syndrome occurs following plaque rupture due to thrombus formation from the coagulated blood that block the circulation. On the other hand, higher Th2 activity leads to a more stable plaque and less susceptibility to rupture.

FIGURE 2-5. Plasma lipid transport in hypertriglyceridemia
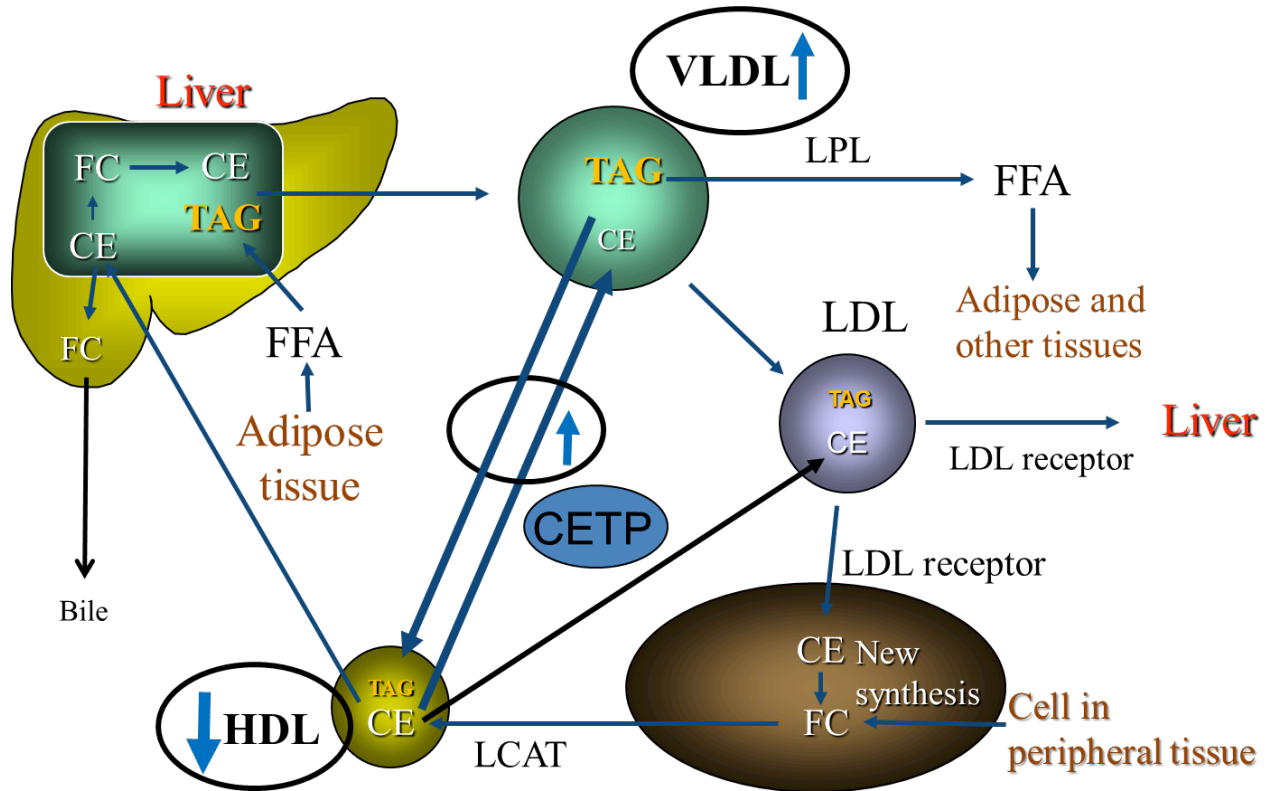
(Source: Barter 2005 [63])

Abbreviations: CE, cholesterol ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FFA, free fatty acid; HDL, high density lipoprotein cholesterol, LCAT, lecithin cholesterol acyl transferase; LDL, low density lipoprotein cholesterol; LPL, lipoprotein lipase; TAG, triacylglycerol; VLDL, very low density lipoprotein cholesterol.

Note: This figure shows overview of lipoprotein metabolism with a highlight on dyslipidemia in CKD/ESRD. In the normal metabolism, the liver secretes VLDLs that rich in TAG. LPL hydrolyze TAG to FFA and promotes extrahepatic cellular uptake. In CKD/ESRD, dyslipidemia is highly prevalence characterized by delayed TAG rich lipoproteins catabolism (i.e. ↑ VLDL/IDL) and low HDLC due suppressed apoA1 production, increased CETP, and reduced LCAT activity.

FIGURE 2-6. Proposed treatment strategies to treat inflammation and minimize risk for CVD in hemodialysis patients

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I. Evaluate and treat intercurrent events and co-morbidities that cause inflammation

- Volume overload
- Infectious complications
- Periodontal disease
- Inflammatory diseases
- Silent ischemic heart disease

⬇

II. Evaluate and handle potential dialysis related cause of inflammation

- Central dialysis catheter
- Unpure dialysate
- Thrombosed fistula or graft
- Volume overload
- Bioincompatible membrane
- Infection of dialysis access

⬇

III. Consider possible anti-inflammatory treatment strategies

Non-specific Immuno-modulation	Nutritional Intervention	Physical training	Anti-cytokine therapy (future treatment)
- Statins - Vitamin D - ACEs / ARBs	- Omega-3 fatty acids - Vitamin E - Soy Isoflavones - Genistein		- IL-1 receptor antagonist - Antibodies against TNF

(Source: Carrero & Stenvinkel 2010 [6])

FIGURE 2-7. Omega-3 molecular structures

$C18:3^{cis\Delta 9,12,15}$

ALA

$C20:5^{cis\Delta 5,8,11,14,17}$

EPA

$C22:6^{cis\Delta 4,7,10,13,16,19}$

DHA
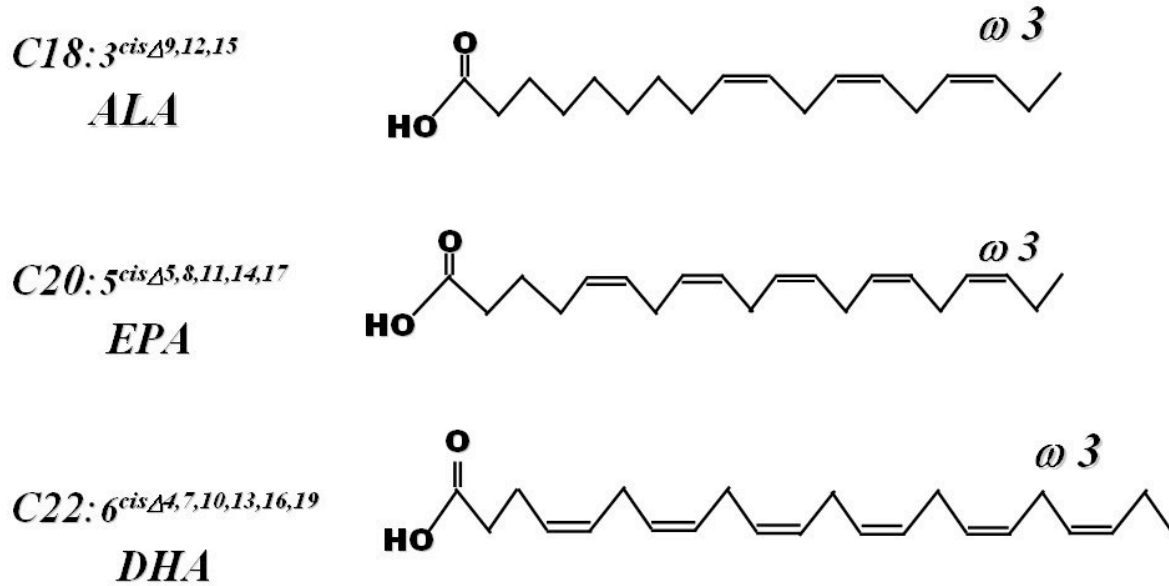
Abbreviations: ALA, α-linolenic acid; EPA, eicosapentanoic acid; DHA, docosahexanoic acid.

Note: This figure shows omega-3 fatty acids molecular structures – ALA (C18:3), EPA (C20:5) and DHA (C22:6). Omega-3 fatty acids have double bond beginning at the third carbon atom from the end of the carbon chain.

FIGURE 2-8. Different effects of omega-3 and omega-6 in relation to eicosanoids
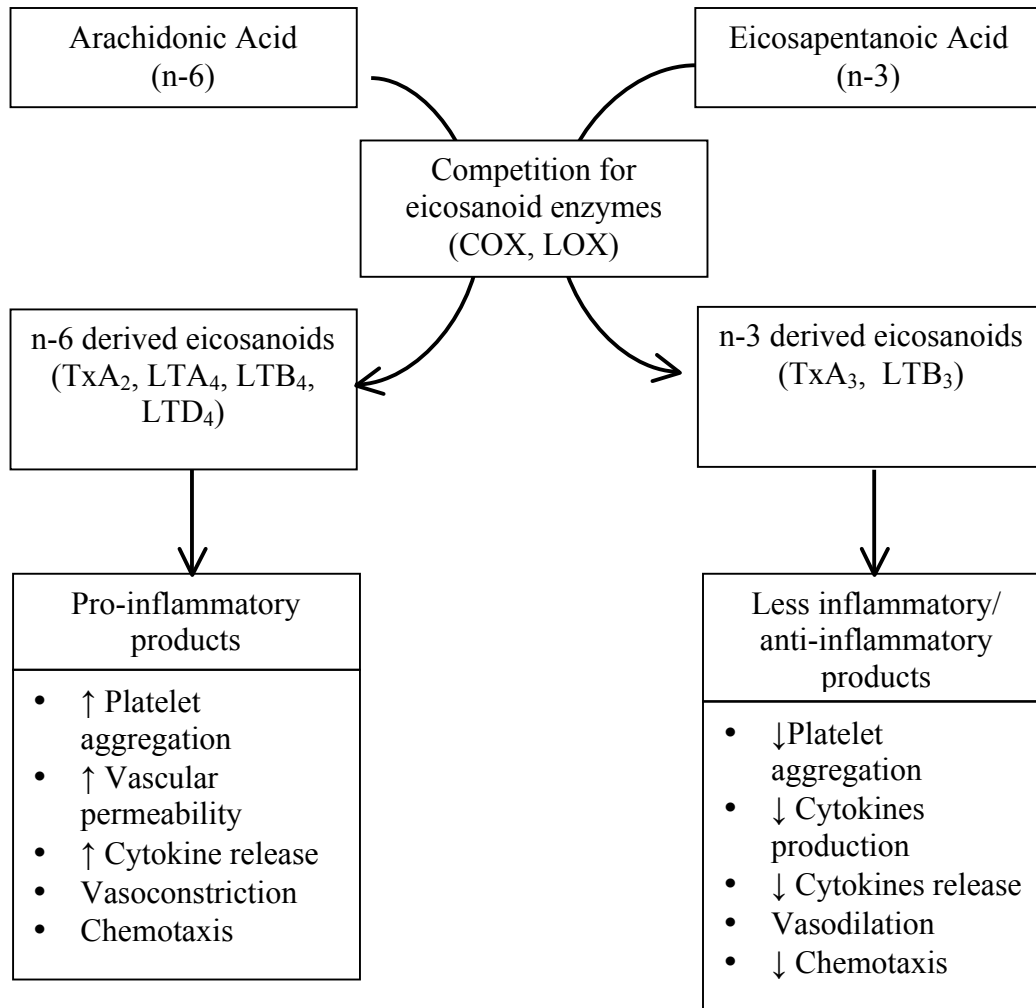
(Source: Friedman & Moe 2006 [92])

Abbreviations: COX, cyclooxigenasae; LOX, lipoxygenase; TxA, thromboxane; LT, leukotriene.

Note: This figure shows synthesis of eicosanoids from omega-6 and omega-3 fatty acids. Eicosanoids are a group compounds that exert a complex control particularly in the intensity and duration of inflammatory response. Eicosanoids derived from omega-6 are proinflammatory by promoting platelet aggregation, vascular permeability and cytokines release. On the other hand, eicosanoids produced from omega-3 is less inflammatory in nature. Because eicosanoids derived from omega-3 use the same enzymatic pathway as those derived from omega-6, high concentration of EPA results in a shift towards non-inflammatory eicosanoids.

FIGURE 2-9. Vitamin E molecular structures
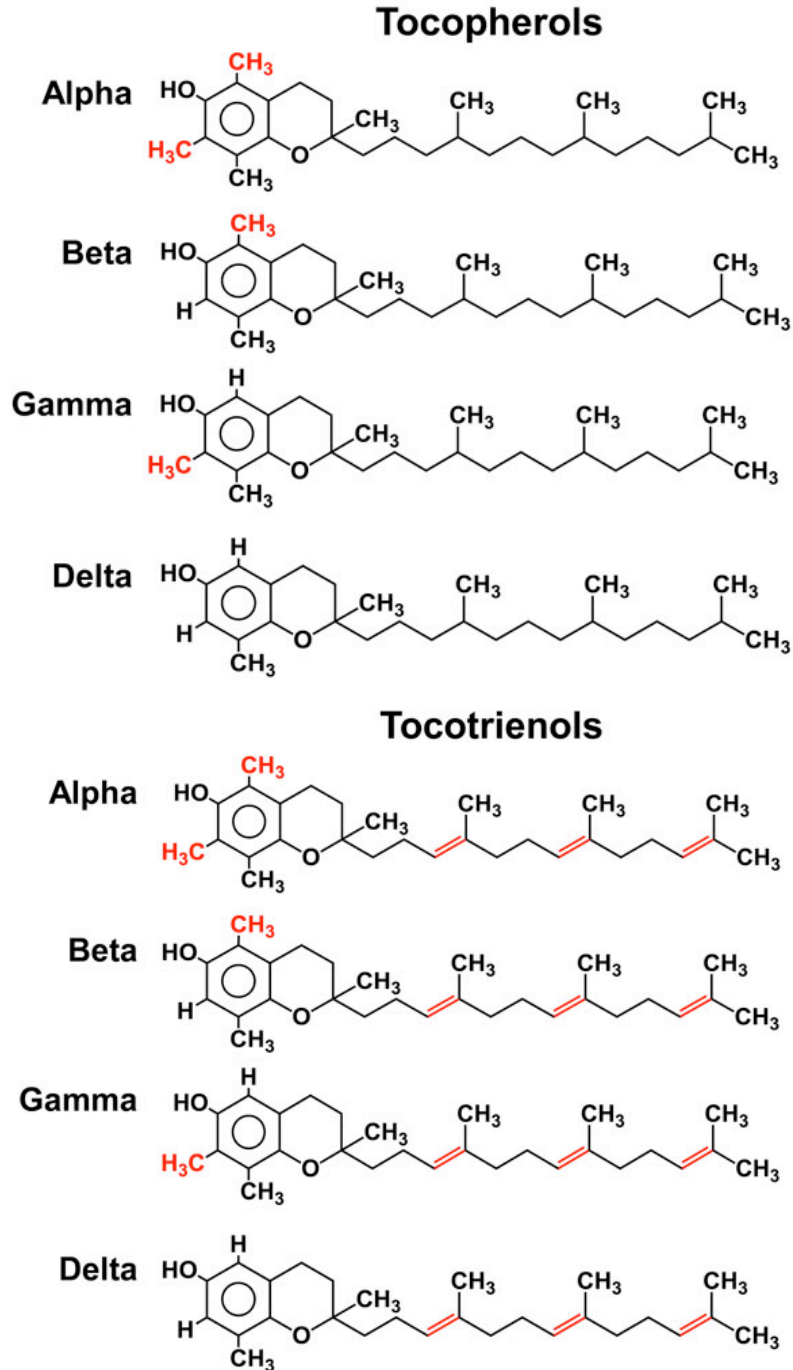
(Source: Aggarwal et al 2010 [33])

Note: This figure shows eight known vitamin E isomers, named as α-, β-, δ-, γ- tocopherol (TT) and tocotrienols (TP) based on the number of methyl group in the chromanol ring. TT are different from TP due to the present of unsaturated chain rather than saturated.

FIGURE 2-10. Dietary sources of tocotrienols
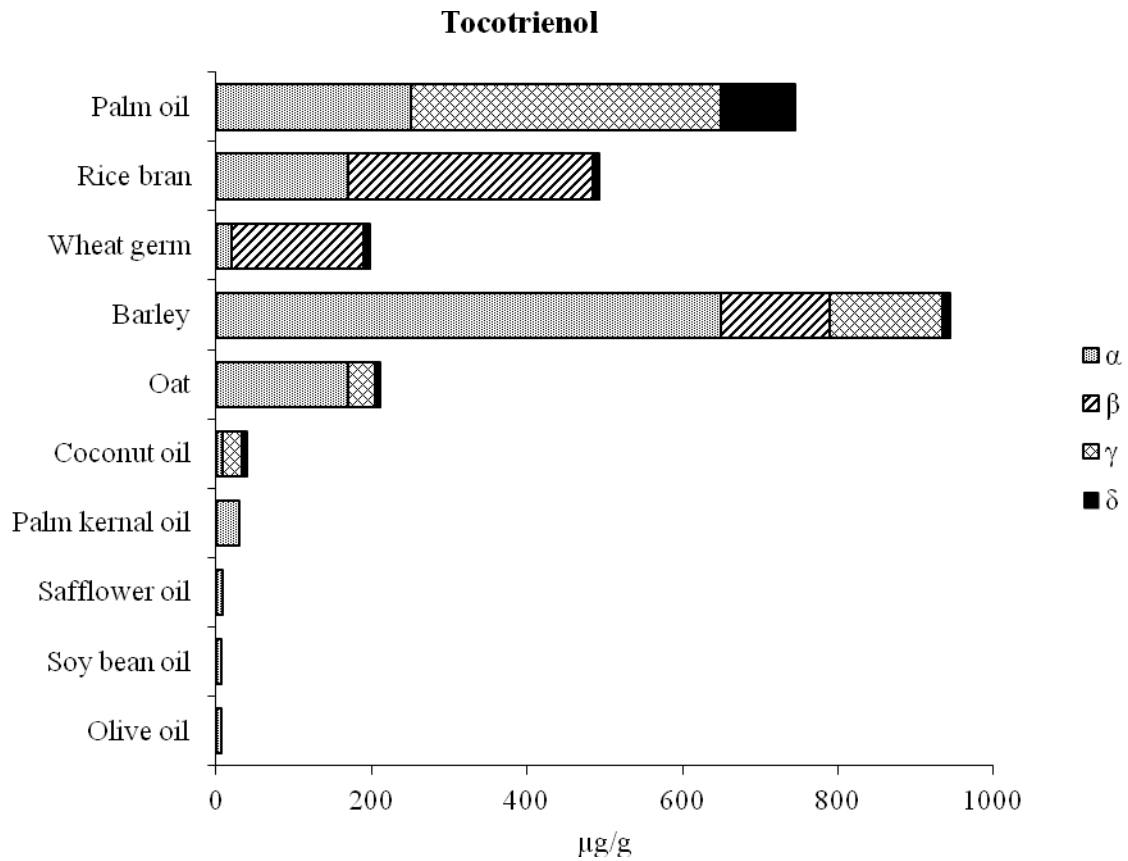
(Adapted from: Aggarwal et al 2010 [33])

Note: This figure shows natural dietary sources of tocotrienol, expressed as μg per gram of the item. Tocotrienol is abundant in palm oil, rice bran, barley, oat and wheat germ while safflower, soybean and olive oil are minimally contain tocotrienol.

FIGURE 2-11. The role of tocotrienols in suppression of the synthesis of pro-inflammatory eicosanoids from arachidonic acid.
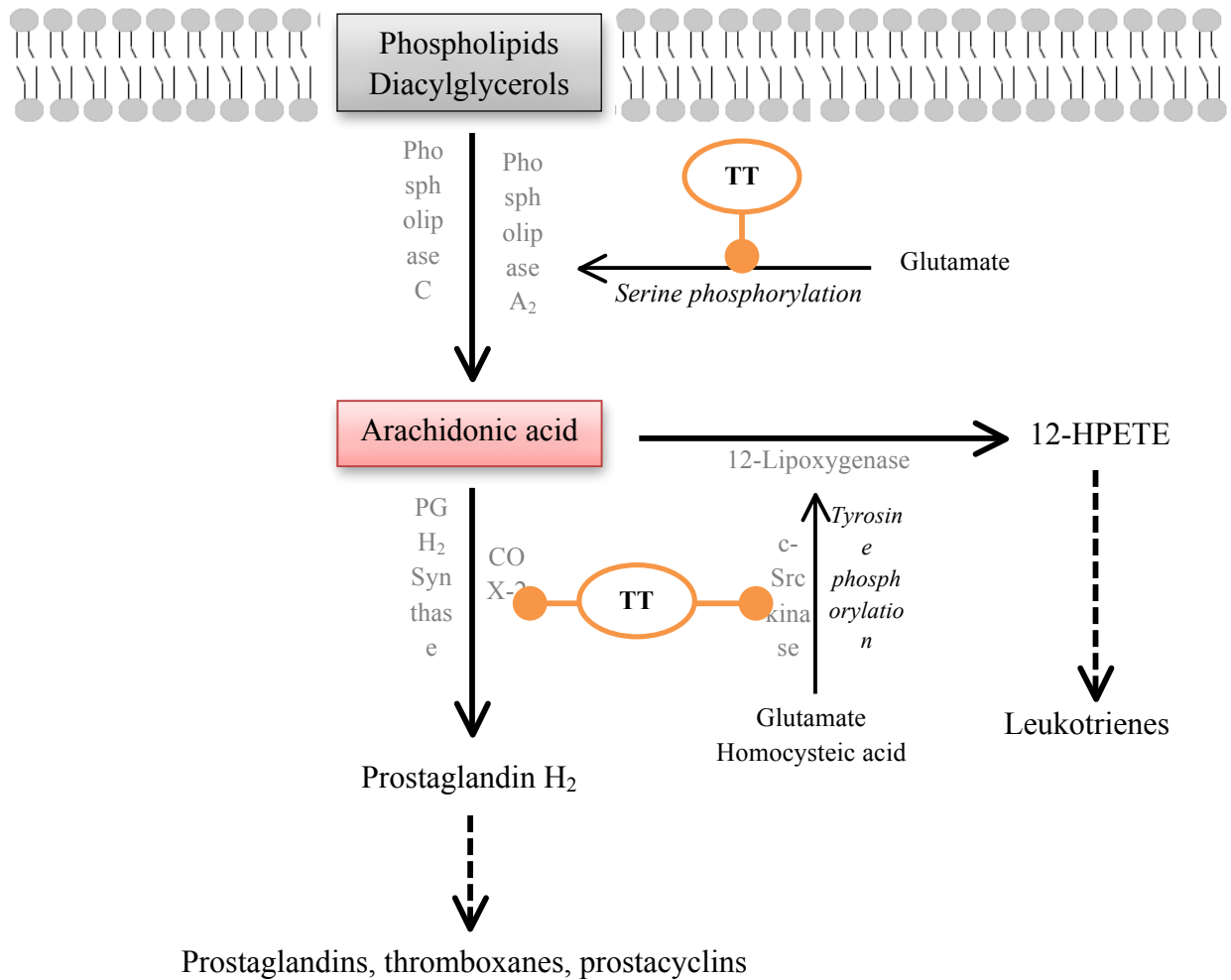
(Adapted from: Frank et al 2012 [116])

Abbreviations: TT, tocotrienols; 12-HPETE, 12-hydroperoxy-eicosatetraenoic acid; COX-2, cyclooxygenase-2.

Note: This figure shows the role of tocotrienols in alleviating production of pro-inflammatory eicosanoids from arachidonic acids. The first pathway is through inhibition glutamate-induced production of phospholipase A_2, which is the enzyme that catalyzes production of arachidonic acid from the cell membrane phospholipids. The second pathway involved inhibition of COX-2 and c-Src kinase resulting in reduced production of pro-inflammatory eicosanoids.

<center>**CHAPTER III**</center>

<center>**METHODOLOGY**</center>

General study design

This dissertation is comprised of three experiments from two different studies. General aim of these studies is to document the effects of supplementation with anti-inflammatory, lipid altering and antioxidant agents (i.e. omega-3 fatty acids and tocotrienols) in hemodialysis patients. These studies were designed as randomized, double-blinded, parallel, placebo-controlled trials using the same cohort of patients undergoing chronic hemodialysis at Great Lake Dialysis Clinic, Detroit MI. There was seven months wash out period between the two studies (**Figure 3.1**).

In the first experiment (**Specific Aim 1**), chronic hemodialysis patients were supplemented with omega-3 fatty acid for 24 weeks to evaluate the feasibility of the supplementation using 'directly observed treatment' and to document the global effects omega-3 on nutritional, lipid profiles and inflammatory markers. Following the proven feasibility of the study using 'directly observed treatment', second study (**Specific Aim 2**) was conducted using similar study design but with some modification to improve compliance and delivery of effective doses to the subjects. In the second study, HD cohort was supplemented with tocotrienols-rich fractions (TRF) for 16 weeks to evaluate the global effects of TRF on nutritional status, lipid profiles, inflammatory markers and oxidative status. Further investigation was carried out to elucidate the effect of TRF on lipid metabolism following positive results on lipid profiles. In the third experiment, the author investigated the changes in patients' metabolomics profile following supplementation (**Specific Aim 3**).

This chapter will describe general methods that were the same between the omega-3 and TRF studies. Specific methods/assays for each study will be described in specific chapter for the particular study (Chapter IV, V and VI).

Ethics and human subjects issues

The Omega-3 study (Specific Aim 1) recruited 63 patients from the Great Lake Dialysis Clinic. The protocol for the Omega-3 study was approved by the Human Investigation Committee of Great Lake Dialysis Clinic (IRB# 00007308) and Wayne State University (HIC #125709A). Informed written consent from the patients was obtained beforehand. Similarly, the protocol for the TRF study (Specific Aim 2a/b) was approved by the Human Investigation Committee of Great Lake Dialysis Clinic (IRB #00007308) and Wayne State University (IRB# 067411A). The study recruited 81 patients of the same cohort and informed consents were obtained from the patients before the study start. Any serious adverse events (e.g. death, inpatient hospitalization, a life-threatening adverse experience, a persistent/significant disability) or unexpected adverse experiences related to the use of these supplements were promptly reported to the Human Investigation Committee using the specify form within five working days.

The Great Lake Dialysis Clinic is an outpatient dialysis facility that is affiliated with Delta Renal Group. The dialysis facility has 24 hemodialysis stations. The hemodialysis patients are distributed equally among seven hemodialysis shifts. Each patient attends hemodialysis session either on Monday-Wednesday-Friday or Tuesday-Thursday-Saturday rotation at a designated time. Each rotation day comprised of three dialysis session namely; Shift 1 runs from 5.15 am to 8.45 am, shift to from 10.15am to 1.45pm and night shift hours of operation are from 5 pm to 4.30 am. Hemodialysis patients are under exclusive care of the nephrologist and a

certified nurse-practioner. A full time registered dietitian provides routine nutritional care and dietary counseling to those patients. Anemia management is performed by the clinical director of the clinic.

General aspects of collection and handling of blood samples

In general, blood was collected from the patients prior to the dialysis session into two 10mL Vacutainer tubes with EDTA and heparin preservatives (BD, Franklin Lakes, NJ, USA). Blood samples were transported to Wayne State University's laboratory within 2 hours after the collection. Additional blood was collected into tubes without anticoagulant for standard renal profiles measurement (ser. Alb, Hb, Kt/V) and were sent to an external laboratory (Ascend Clinical Laboratory Services, Redwood City, CA, USA). Blood samples collected in the EDTA tubes were designated for analyses of lipid profiles and NFκB while lithium heparin tubes were used for analyses of oxidative status and inflammatory markers. Plasma was separated by centrifugation at 2800 rpm for 20 minutes at 4°C (GS-6KR Centrifuge, Beckman-Coulter, USA) and aliquots were stored at -80°C until further analysis. Blood sample for nuclear factor kappa B (NFkB) were processed immediately to obtain mononuclear cell extract using Ficoll-Paque method (GE Health Care, Piscataway, NJ, USA), and the nuclear extract were kept at -80°C until use.

Anthropometry measurements

The anthropometrics measurements carried out in both studies included estimated dry weight and height at the baseline and the end of the study. Height was measured to the nearest 0.1cm (Tanita Wall Mounted Height Rod, Tanita, USA). Body weight was measured to the nearest 0.1kg after each HD session (Tronix Flush-Mounted In-Floor Scale, Scale-Tronix, USA). Body mass index

(BMI) was calculated from the measurements of weight and height using Quetelet 's Index [BMI (kg/m2) = weight (kg) / height (m2)][170] and compared to WHO (2000) BMI classification chart [171].

Biochemical analyses

Biochemical analyses performed in both studies included 1) Renal profiles (ser. Alb, Hb, Kt/V) (performed by external laboratory) 2) Lipid profiles (TC, TAG, HDL-C) and 3) Inflammatory markers (C-RP, NFκB). Brief descriptions of each assays performed in these studies are described as follow:

Lipid profiles

TC and TAG in the plasma was determined using commercial enzymatic assay per manufacturer protocol (Pointe Scientific, Canton, MI). Briefly, 10μL of unhemolyzed plasma samples were transfer into test tubes, followed by addition of 1.0mL of TC/TAG reagent (pre-warmed to 37°C) and gently mixed by vortexing. The tubes were incubated for 10 minutes in the dark and the final color intensity of the solution was recorded at 500nm within 30 minutes (DU 640 Spectrofotometer, Beckman-Coulter, USA). TAG concentration for each sample was calculated by comparing to the standard absorbance (abs unknown/ abs standard X concentration standard) and the results were expressed as mg/dL. All samples were assayed in duplicate.

HDL-C was measured in the supernatant fraction following precipitation of ApoB containing lipoprotein using dextran sulfate and magnesium ions (Pointe Scientific Inc., Canton, MI). In short, 150μL of plasma was transferred to microtube followed by the addition of 30μL of Mg/Dextran Sulfate and 10 minutes incubation at room temperature. The solution was spun at 10,000 rpm for 5 minutes to precipitate ApoB containing lipoprotein. Supernatant fraction

(50µL) was transferred to the test tubes in duplicates followed by the addition of 1.0mL of pre-warmed TC reagent. After 10 minutes incubation, the color intensity was measured using spectrophotometer at 500nm. HDL-C concentration for each sample was calculated by comparing to the standard absorbance (abs unknown/ abs standard X concentration standard) and the results were expressed as mg/dL. LDL-C was calculated using the Friedewald's equation by difference (LDLC = TC minus HDLC minus TAG/5)[174].

Inflammatory markers

CRP (Cayman Chemical, Ann Arbor, MI) levels were measured using commercial kits based on enzyme-linked immunoabsorbent assay (ELISA) method as per the manufacturer's protocol (Cayman Chemical, Ann Arbor, MI). Plasma samples were diluted in the assay buffer into 1:15,000 and 100µL of each sample was placed into 96-wells microplate that has been coated with a monoclonal antibody specific for human CRP and were incubated for an hour. The plate was then rinsed for four times with wash buffer before addition of HRP-labeled CRP monoclonal antibody. Two antibodies were added and formed a sandwich by binding to different location of CRP molecule. CRP concentration in the plasma samples were measured at 450nm after addition of chromogenic substrate TMB (tetramethylbenzidine) that formed a distinct yellow color. Standard curve was prepared using a known concentration of CRP with data being expressed as mg/L. A general principle of sandwich ELISA method is presented in **Figure 3-2**.

NFκB assay was performed based on three steps: 1) Isolation of mononuclear cells from the blood, 2) Extraction of nuclear and cytoplasm extract, and 3) Measurement of NFκB expression/concentration in the nuclear extract. Isolation of the mononuclear cells from the blood was performed using Ficoll-Paque PLUS solution (GE Healthcare Life Science, Pittsburgh, PA,

USA). Briefly, 5mL of fresh blood that were collected in EDTA tube were diluted with an equal volume of 1 X phosphate buffer saline solution. The diluted blood were then transferred into a 15mL Corning centrifuge tube (Corning Life Science, Tewksbury, MA, USA) containing 10 mL of Ficoll-Paque solution by carefully overlaying it using 9" Pasteur pipette (Thermo Scientific, USA). This was followed by a centrifugation at 1,400 rpm, 20°C for 30 minutes to separate peripheral blood mononuclear cells (PBMC). PBMC was harvested using Pasteur pipette, transferred into a new 15mL Corning centrifuge tube, followed by an addition of three volumes of 1 X PBS. The solution containing PBMC was centrifuge again at 1,000 rpm, at 4°C for 10 minutes to eliminate contaminant (e.g. platelet). The washing processes were done two times before the PBMC were harvested and stored at -80°C until further use. Nuclear and cytoplasm extract were obtained from PBMC using kits purchased from PIERCENET (Thermo Scientific, Rockford, IL, USA) per manufacturer's protocol. For a 40mg packed cell volume, 400μL of cytoplasmic extraction reagent I (CER-I) was added into the cell pallet followed by a vigorous vortexing for 15 seconds and incubation on ice for 10 minutes. CER-II solution (22 μL) was added into the tube followed by vortex and centrifugation for 5 minutes at 16,000g, 4°C. The cytoplasmic extract was harvested from the supernatant while the insoluble pallet was used to extract the nuclear extract by the addition of the nuclear extraction reagent (NER). Protein content of the nuclear extract were determined based on Bradford's method using a commercial reagent purchased from PIERCENET (Thermo Scientific, Rockford, IL, USA) per manufacturer's protocol. Upon the quantification of the protein content, each sample was standardized for the protein concentration by a dilution with NER solution. NFκB p65 in the nuclear extract was determined using TransAM NFκB (Active Motif, Carlsbad, CA, USA) based on ELISA method as discussed earlier on.

Statistical analysis

Upon the completion of the study, data were keyed-in in Microsoft Excel, checked for their integrity and missing values before transferring into SPSS. Statistical analyses were carried out using SPSS version 16.0 (IBM, Chicago, IL). Continues data were presented as mean±SD (in tables) and mean±SEM (in figures), while data that were not normally distributed were presented as median±IQR. Categorical data were presented as absolute number and percentage.

Independence t-test were applied to normally distributed continuous data to check differences in mean between the two groups, while paired t-test were used to check differences between time points. For continuous data that were not normally distributed, equivalent non-parametric tests were used to test differences in mean of the two groups and between the time points. Chi-square test was used to test differences in categorical data. Pearson-correlation coefficient test was used to evaluate correlation of the studies' parameters.

FIGURE 3-1. General study design.
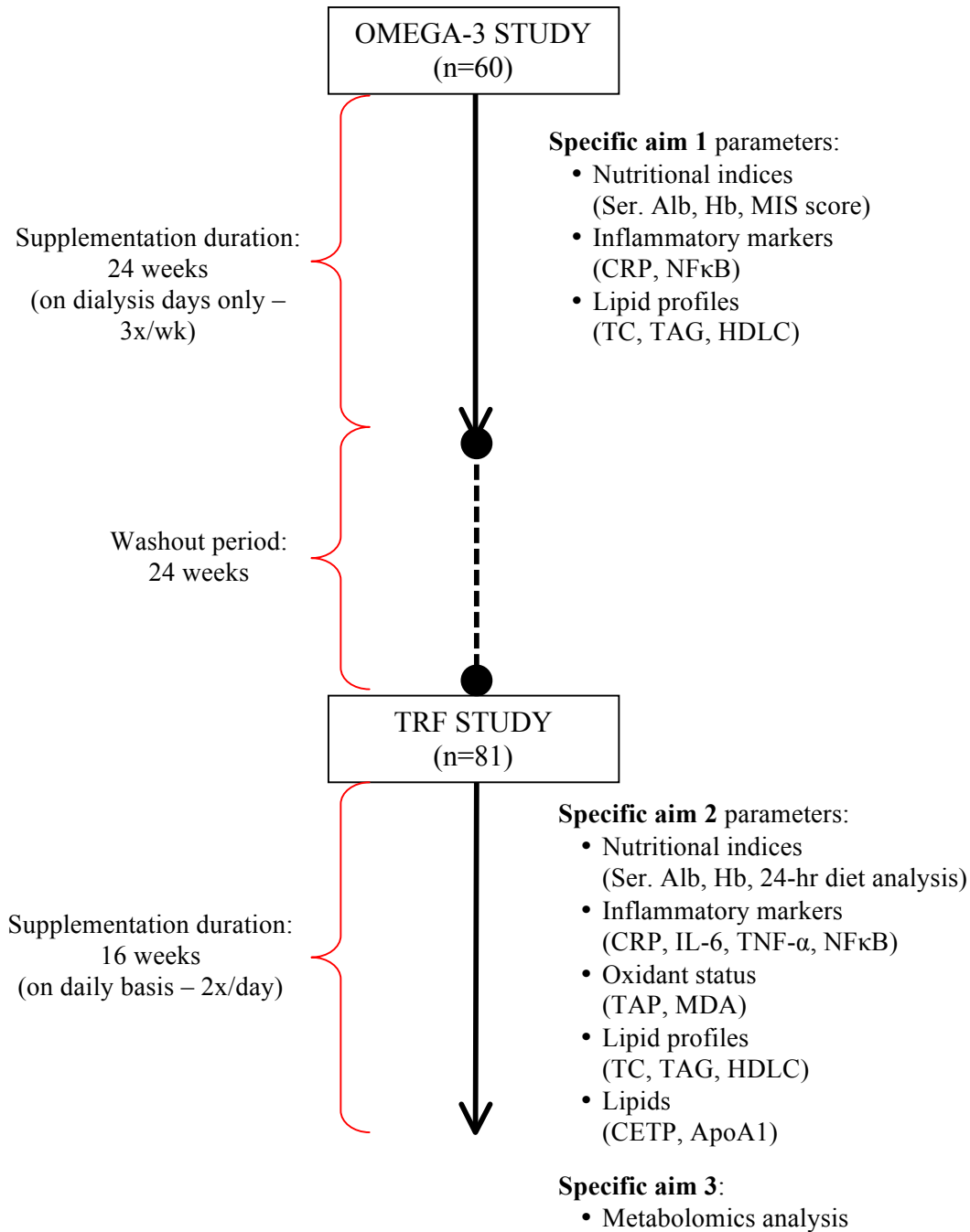
Abbreviations: Ser. Alb, serum albumin; Hb, hemoglobin; MIS, malnutrition-inflammation score; CRP, C-reactive protein; NFκB, nuclear factor kappa B; IL-6, Interleukin-6; TNF-α, tumor necrosis factor alpha; TAP, total antioxidant power; MDA, malondialdehyde; TC, total cholesterol; TAG, triacylglycerol; HDLC, high-density lipoprotein cholesterol; CETP, cholesteryl ester transfer protein; ApoA1, apolipoprotein A1.

FIGURE 3-2. Principle of sandwich ELISA
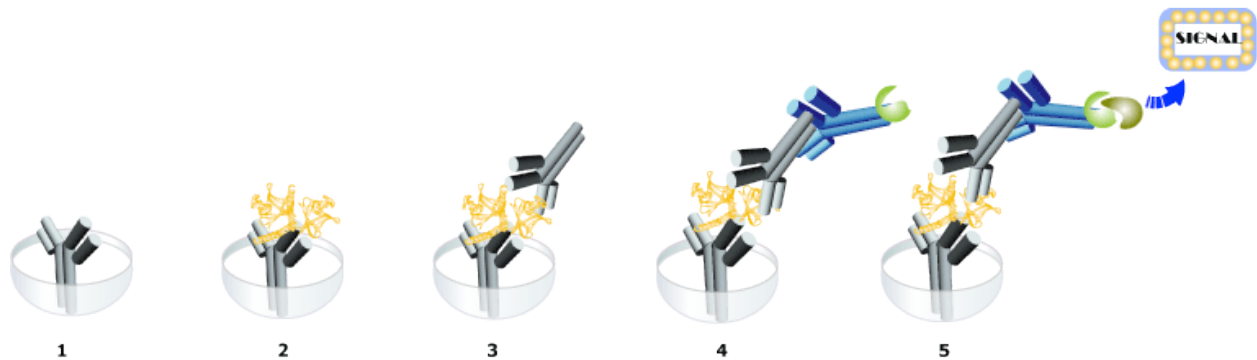
(Illustration by: Genway Biotech Inc, 2013)

1. A 96-well microplate is pre-coated with desire antibody by the manufacturer (e.g. human CRP, IL-6).

2. Plasma sample is added to the each well in the microplate in duplicate. Any antigen found in the plasma is bound to the capture antibody.

3. Biotin labeled detection antibody is bound to the antigen.

4. HRP is added and binds the biotin labeled antibody.

5. TMB substrate is added and converted by HRP into detectable color solution. The concentration of the antigen is quantified using spectrophotometer.

Note: This figure shows principle of sandwich ELISA that has been used to measure CRP, IL-6, TNF-α and NFκB in this project.

CHAPTER IV

SPECIFIC AIM 1:

EFFECTS OF PROTEIN AND OMEGA-3 SUPPLEMENTATION ON NUTRITIONAL STATUS, LIPID PROFILES AND INFLAMMATORY INDICES

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Introduction

Cardiovascular disease (CVD) represents the major source of morbidity and is a leading cause of death in uremic patients on chronic hemodialysis [164]. From non-traditional cardio-vascular disease risk factors in dialysis patients, a malnutrition-inflammation complex syndrome has been postulated to play a role in the etiology of premature CVD in hemodialysis patients [7]. Thus, there is considerable interest in finding appropriate interventions for the malnutrition-inflammation complex syndrome (MICS) in this population.

Protein energy malnutrition (PEM) is highly prevalent in dialysis populations which negatively impacts prognosis. PEM is implicated in a complex syndrome caused by nutritional and non-nutritional factors such as deficient food ingestion secondary to uremia, dietary restriction, chronic inflammatory state and increased catabolism related to treatment modalities, nutrients loss in dialysate and metabolic acidosis [74]. Numerous studies have demonstrated that protein supplementation alone may improve protein metabolism, nutritional parameters and therefore improve clinical outcomes [176-178]. Meanwhile, causes of inflammation are multifactorial comprising of dialysis and non-dialysis factors such as exposure of blood with

dialyzer membranes or tubing, infection on vascular access, reduced antioxidants and increased oxidative stress [74, 179]. Therefore, in order for a nutritional intervention to be most effective, it should be targeted at improving protein status and have anti-inflammatory properties to improve inflammatory status [159].

There is a growing scientific interest on the application of omega-3 fatty acids as a means to prevent CVD in dialysis population. Omega-3 fatty acids have been postulated to reduce a pro-inflammatory response, confer antithrombotic properties, improve lipid levels particularly triglycerides and improve endothelial functions in several studies [98, 104, 180, 181]. To date, there is no well-established recommendation on omega-3 fatty acid supplementation for the dialysis population despite the fact that the dialysis population has lower omega-3 intake [32] and plasma levels [102]. Some of the studies published used supraphysiological doses of omega-3 (>3 g/d) which required the high number of capsules and may be difficult to consumed long term [92]. Moreover, there may be barriers to compliance with this type of nutritional intervention especially when the study population includes non-Caucasian races, low-income groups and unemployed [182]. Some of the clinical studies only rely on subjective method such as pill counting to measure compliance [109, 161] which could be the important limitation for the likely type of non-compliance population.

Therefore, we performed a pilot study to investigate the technical feasibility of "directly observed treatment" of nutritional supplementation administered during regular dialysis sessions. Secondary outcomes included observations on nutritional and inflammatory status of hypoalbuminemic patients undergoing hemodialysis. We hypothesized that a combination of protein and omega-3 supplementation would be more effective than protein supplementation alone in improving nutritional and inflammatory status.

Methods

Patients

One hundred-and-five patients who were undergoing routine hemodialysis treatment in the Great Lake Dialysis Clinic in Detroit, Michigan were screened. Selection criteria for patients included serum albumin levels not meeting the outcome goal of K/DOQI guidelines [183] ($\leq$3.9 g/dL), age greater than 18 years old and ongoing dialysis treatment for at least 3 months prior to the study. Patients who were residents of nursing homes or receiving intradialytic parenteral nutrition, tube feeding, undergoing 8 hours of dialysis treatment, nocturnal dialysis, serum albumin > 3.9 g/dL, or fish allergies were excluded.

Study design and procedures

The present study was approved by the Human Ethics Committees of Wayne State University and the Human Investigation Committee of Great Lakes Dialysis. Informed written consent was obtained from all patients. Eligible subjects (n=63) were randomized into two groups - placebo +protein supplement (Placebo, n=32) and omega 3 + protein supplement (Omega3, n=31). Patients, care givers and investigators were blinded from group assignments. This study lasted 6 months.

Nutritional intervention and compliance

The nutritional intervention provided comprised of two components. Both treatment groups received a blend of 30 mL liquid protein supplement (Proteinex) (Llorens Pharmaceuticals Inc, Miami, FL) in 60 mL of apple juice providing 100 kcal, 18g protein and 8 gram carbohydrate [specifically, 30mL of Proteinex provides 18g of protein hydrolysate (72 kcals), with 1440mg of

L-Arginine, 540mg of L-Leucine, 420mg of L-Phenylalanine, 132mg of L-Histidine, 780mg of L-Lysine, 360mg of L-Isoleucine, 132mg of L-Methionine and 66mg of L-Tryptophan]. Additionally, subjects were given 4 capsules of either omega-3 or placebo, formulated by Twin Rivers Technologies (MA, USA). Each one of the omega-3 capsule contained 150 mg of DHA and 450 mg of EPA (total DHA 600 mg, total EPA 1800 mg), while the other capsule was a placebo comprised of olive oil. Analysis of omega-3 and placebo capsule is presented in Table 1. Both protein and capsules were given to the patients 3 times a week after their regular dialysis session for a total duration of 6 months. Compliance was ascertained by administering the supplements during the patient's dialysis session with close monitoring by nurses. Tolerability and side effects for supplements were recorded by registered nurses and hospitalization rates were also monitored.

Blood sampling and laboratory measures

The study flow chart is shown in Figure 1. Approximately 20 mL of fasting blood sample were taken prior to the dialysis session. Plasma samples intended for lipid analysis were isolated from blood by centrifugation at 3000 rpm for 20 minutes at 4oC and were kept at $4^{o}C$ to be analyzed on the following day. Blood samples for NFκB were processed immediately to obtain mononuclear cell extract using Ficoll-Paque method [184] and the extract was kept at -80oC until further analysis. Serum samples for common renal profiles (serum albumin, BUN, creatinine, hemoglobin, etc) were sent to Satellite Laboratory Services (Redwood City, CA) for analysis and were measured using standard automated laboratory techniques.

Lipids analysis

Total cholesterol, HDL cholesterol and triacylglycerol (TAG) in the plasma were determined by enzymatic reaction using kits purchased from Pointe Scientific Inc. (Canton, MI). LDL

cholesterol was calculated using the Friedwald equation (LDL-C = TC minus HDL-C minus TAG/5) [174]. LDL-C to HDL-C ratio was calculated by dividing LDL-C by HDL-C values.

Inflammatory markers

Serum CRP was analyzed by an independent laboratory (DMC Laboratory, Detroit, MI) using immunoturbidimetric method. Activated NFκB was measured from the nuclear extracts using TransAm chemiluminescent (p65) kit purchased from Active Motif (Carlsbad, CA) using the procedure described by the manufacturer.

Nutritional indicators

Serum albumin was measured by bromcresol green method using standard automated laboratory techniques. Normalized protein nitrogen appearance (nPNA) was calculated according to K/DOQI guidelines (2000) [183]. Malnutrition-inflammation score (MIS) questionnaire [89] was also administered prior to (baseline), during (month-2) and at the end of the study (month-6). MIS is a quantitative scoring system that comprised of 10 components related to patients' medical history, physical exam, body mass index and laboratory parameters, which has been reported to be a useful tool to predict dialysis outcome, malnutrition and inflammatory status [185-187]. MIS was performed and calculated by a single person, a registered dietitian, to minimize inter-observer variability.

Statistical analysis

The minimum sample size for each group was n=31, which was estimated at a power of 80% and α=0.05 for a two-arm parallel study to detect a 0.2g/dL difference in serum albumin. Results were analyzed using intention to treat principle. All study participants were maintained in the treatment groups to which they were randomized regardless of post-randomization withdrawal

and any missing values reported accordingly. Categorical data are reported as number and percentage, while continuous data are reported as mean±SD or as otherwise stated. Independent t-test was used to determine differences in the variables of interest between the two groups at baseline. Difference between baseline and month-2 and between baseline and month-6 for each group were tested using paired t-test. Data that were not normally distributed were tested using non-parametric test. Statistical significance was set at α=0.05. All statistical tests were performed using SPSS v. 16. As there was no significant difference between baseline and the 2-month values in any of the parameters measured, these are not discussed further (data not shown).

Results

Demographic profiles

Sixty-nine patients met the inclusion criteria, and 63 agreed to participate. Subjects were randomized based on albumin levels, and with the exception of gender distribution, there were no significant difference in age, duration on dialysis, BMI and prevalence of existing co-morbid (diabetes mellitus) or indicators of dialysis adequacy (Kt/v) between the two groups (**Table 2**). The study population was homogenously comprised of African-American ethnicity with no difference in income between groups.

Tolerability, side effects and compliance

Over the course of the study, four patients passed away due to a) cerebrovascular accident (one subject, P group), b) diabetic infection (one subject, Omega3 group) and c) CVD (two subjects, one from each group). There were no other adverse events reported (i.e. a life-threatening adverse experience, inpatient hospitalization or prolongation of existing hospitalization, or a persistent or significant disability/incapacity), over the course of the study. No discernible

"fishy" smells were reported by any of the patients with regards to capsule assignment. Two patients complained they couldn't swallow the pills (P group) and withdrew from the study (**Figure 1**). Twenty-one patients (33%) were able to take 100% of the supplement provided (no missing day) during the 6-month study period. Sixty-seven percent of the patients (n=22 for Omega3 group and n=21 for P group) were able to take greater than 80% of the supplements. The principal reason for "lack of compliance" was subjects who missed their scheduled dialysis sessions.

Effects on lipid profiles

One subject was excluded from the analysis due to high TAG levels (478 mg/dL) as Friedwald equation for LDL-C estimation is not reliable when TAG levels exceed 400mg/dL [188]. Total cholesterol was significantly reduced over the 6 months intervention in both groups (p<0.001) (**Table 3**). At the end of 6 months, the reduction in TC in the Omega-3 group (-37±32 mg/dL) was approximately fifty percent greater than the reduction seen in the P group (-21±26 mg/dL, p=0.057). While HDL-C levels were increased at month-6 compared to the baseline in both groups (t=-4.077, p=0.000 and t=-3.410, p=0.004, respectively), there was no significant difference between groups (p=0.531). LDL-C was reduced in both groups (Omega3 group: t=6.083, p=0.000, P group: t=4.200, p=0.000) at month-6 as compared to the baseline, however, no significant differences between the two groups were noted at the end of the study (p=0.092). The LDL-C/HDL-C ratios were significantly improved in both groups as compared to baseline (Omega3 group: t=5.875, p=0.000, P group: t=4.683, p=0.000). After 6 months, the Omega3 group had a significantly larger reduction in LDL-C/HDL-C ratios as compared to the P group (-1.3±1.1 vs -0.7±0.8, p=0.043). As compared to baseline, TAG levels did not change in the P group (p=0.561), while there was a tendency for a decline in the Omega3 group (p=0.064)

Effects on nutritional parameters

Serum albumin and other nutritional indices are presented in **Table 4**. Serum albumin at the end of the study was comparable to the values observed at baseline in both the Omega3 (t=-1.895, p=0.070) and P (t=-0.314, p=0.756) groups. Comparison between both groups showed no-significant difference (p=0.295). Similarly, no changes were observed in MIS score, nPNA and BMI following the intervention within or between the groups. Additionally, hemoglobin levels were not changed following intervention in both groups (Omega3 group: t=1.100, p=0.282, P group: t=-0.731, p=0.471).

Effects on inflammatory indicators

The NFκB levels observed after 6 months in the Omega3 and P groups were similar to their baseline values (**Table 5**). Additionally, mean differences (month-6 minus baseline) between Omega3 and P groups were not significantly different (p=0.134). With regards to CRP levels, mean values showed a significant elevation in CRP levels in the P group (t=-2.158, p=0.040) but not the Omega3 group (t=-0.582, p=0.565) over the 6 months. However these differences were not apparent when median CRP values were compared. Additionally, there was a significant inverse association between CRP and albumin levels across both groups (r= -0.261, p=0.049).

Discussion

Our study tested the potential of a combination of protein and omega-3 supplementation to improve nutritional and inflammatory markers in chronic hemodialysis patients. To increase compliance the supplements were administered during patient's routine dialysis session, three times per week ("directly observed treatment"). Directly observed treatment allowed for 2/3rds of the subjects to receive more than 80% of the stated overall dose over a six months period. While compliance was excellent in patients who came in for regular dialysis sessions, the

significant comorbidities within this patient population resulted in a large number of missed dialysis treatments (and hence missed supplementation).

We observed that serum albumin, one of the markers of nutritional status, was not improved significantly in either group following the intervention. This finding was in contrast to what has been reported in previous studies [189-191]. This may be explained by the fact that only a small number of patients were able to take all of the supplement provided to them (no missing day) during the study course (n=21, 33.3%). Patients missed days of supplementation mostly due to frequent hospitalization or failed to show up for their routine dialysis treatment, ranging from 1-44 days. Therefore, this could potentially upset the effect of omega-3 and protein supplementation per se. Further analysis on a pool of patients who substantially completed the supplementation course (i.e. taking >80% of the supplements) showed marginal improvement in serum albumin at month-6 as compared to the baseline (3.6±0.3 g/dL vs 3.7±0.3 g/dL, p=0.079) but no improvement were noted in those who took <80% supplements (3.7±0.2 vs 3.7±0.4, p=0.939). Interestingly, nPNA, an indicator of protein intake, remained constant in both group despite protein supplementation. A similar study by Morretti et al. [192] also noted a similar trend, in which there were no changes in nPCR despite improvement in serum albumin in the protein group for the second 6 months.

Reduced protein intake and an increase in inflammatory response are two important factors that lead a decrease in serum albumin [193]. However, Kaysen et al [194] had noted that low serum albumin in dialysis population may be attributed to systemic inflammation rather than nutritional inadequacy as a causative factor per se. In relation to that, we also observed a significant inverse correlation between serum albumin and CRP which may indicate the influence of inflammatory process in serum albumin levels. Friedman and Fadem [195]

suggested that serum albumin should be taken as a marker of illnesses rather than nutrition. This is due to the fact that serum albumin has a strong ability in predicting mortality but rather limited prediction for nutritional status due to significant influence of non-nutrition causes (such as inflammation).

Many of the omega-3 fatty acid supplementation studies in dialysis population have described various potential outcomes such as attenuating dyslipidemia, inflammation and providing cardio-protective effect. Moreover, there is substantial evidence for reducing TAG levels [27, 28, 30] even at a dosage as low as 1.5g/day [29]. As for plasma TAG, we saw a tendency for a reduction in TAG levels in both groups although in the O3PP group statistical significance was marginal (p=0.064). The large variation in the difference in TAG changes observed in the PPP group (-7±61 mg/dL, p=0.56, **Table 3**) could be attributed to one subject with very high triacylglycerols concentrations. Upon removal of this subject the difference in TAG between the 6 month and baseline value was 1±44 mg/dL, p=0.89. Thus collectively these data suggest no change in TAG over 6 months in the placebo group, while there was a tendency for a reduction in TAG in the Omega3 group (-20±44 mg/dL, p=0.064). Previous studies [106, 161] which administered a higher amount of omega-3 per week, with varying percentage of EPA/DHA but with a shorter duration (4 – 8 weeks) also reported no changes in TAG levels. However, Bouzidi et al (2010) reported that a supplementation of 2.1g omega-3 daily for 3 months among CKD patients reduced TAG levels by 48% [196]. However, it is important to note that the baseline TAG levels in their study were much higher than the current study (275±58 mg/dL vs 123±60 mg/dL in omega-3 group). We postulate that, the baseline TAG levels (O3PP: 123±60 mg/dL and PPP group: 104±69 mg/dL) were within the normal levels and thus may not have been sufficiently elevated to achieve a significant reduction. Skulas-Ray et al reported that

the extent of TAG lowering with omega-3 is a function of baseline TAG levels [197]. Omega-3 supplementation resulted in better improvement in LDL-C/HDL-C ratios as compared to placebo which appeared to be due to a greater improvement in non-HDL-C. As for inflammatory markers, our results for CRP were inconclusive. While mean values in the P group increased over the 6 month period, this was not the case in the Omega3 group, in which CRP levels stayed the same. Thus Omega-3, if not effective in attenuating inflammation, may be beneficial in preventing further increases in inflammatory status. However when the data were evaluated using median CRP values, these differences were no longer apparent suggesting the need for a larger sample size to definitively resolve this issue. Only a small number of studies have investigated the effects of omega-3 on inflammatory marker in hemodialysis population. It is important to note that, most of these studies are not comparable due to difference in study design, supplement dosage, EPA/DHA ratios and study duration. Saifullah et al showed that, a supplementation of 1.3 g of oral EPA and DHA daily over the period of 3 months could modestly reduced CRP levels [108]. However, a recent study using a larger dosage (2.08g/day) but a shorter duration (10 weeks) showed no effects on serum systemic inflammation markers (CRP, IL-6, TNFα) and oxidative stress (MDA, TAC) [198]. In a prospective cohort study by Noori et al (2011), the authors showed that, a lower omega-6 to omega-3 ratios (~6) was associated with decreased inflammation and overall mortality in hemodialysis population which indicate the importance of n-6/3 PUFA ratios in the diet [32]. However, our study was not designed to investigate the effects of the ratio *per se*.

NFκB, a key player in pathogenesis of inflammation, is stimulated by pro-inflammatory agents such as cytokines and CRP. Studies have shown that, NFκB activation and subsequent activation of mononuclear cells trigger a process that cause myocardial inflammatory damage in

hemodialysis patients [199, 200]. Interestingly, our data for translocation of activated NFκB to the nucleus showed no significant changes following the intervention within and between both groups even though we saw an increase in CRP levels in the placebo group. The latter may have been due to the fact that the baseline CRP values in the placebo group were lower than those assigned to the omega-3 group.

Limitations of the study

We were restricted in our patient population to one dialysis clinic for this pilot study. While the lipid data suggested benefits of Omega3, the data for inflammatory markers was less clear-cut. One plausible explanation for variation in inflammatory markers in the current study may be related to insufficient dosage/frequency of Omega-3 supplementation as well as compliance (due to frequent hospitalization resulting subjects missing dialysis sessions and therefore supplementation). There are several similar studies that also failed to report significant changes in inflammatory markers [161, 201-203]. Studies that demonstrated a significant effect of omega-3 supplementation on inflammation indices are those with a higher dosage and more frequent administration of the supplement (9.1 to 21 g/week vs 7.2g/week DHA + EPA in the current study) as well as reported better patients' compliance [204-207]. Another factor that could potentially explain the contradictory finding in our study with those studies is baseline levels of inflammatory markers. Omega-3 supplementation appears to be more effective in studies reporting higher baseline CRP (>13.8 mg/dL) than those which did not [109, 198, 204].

Notwithstanding some of the limitations discussed above, ours is one of the few studies to report on the use of "directly observed treatment" of oral nutritional supplementation with protein and omega-3 in a cohort of hemodialysis patients. Our results suggest that "directly observed treatment" is technically feasible in hemodialysis patients and does not disrupt the

normal dialysis schedule. The relatively large number of missed dialysis sessions due to hospitalizations, however, suggests that "directly observed treatment" should be used in the setting of both outpatient and in-patient dialysis sessions in order to optimize compliance. In addition, while emphasizing the huge progressive inflammatory burden in hemodialysis patients over time; this pilot study suggests that "directly observed treatment" with a combination of omega-3 and protein based supplement (as opposed to a pure protein supplement) could have some benefical effects on lipid profile and CRP progression. Further studies using a combination of out-patient "and" in-patient "directly observed treatment" of fish oil based nutritional supplementation are warranted.

Tables and figures

TABLE 4-1. Selected fatty acid composition of omega-3 and placebo capsule.

	Omega-3 (g/100g as FA)	Placebo (g/100g as FA)
C16:0	0.96	13.86
C16:1	0.35	1.44
C17:0	0.14	0.07
C17:1	-	0.11
C18:0	4.66	2.73
C18:1n9	8.88	66.64
C18:2n6	2.00	11.42
C18:3n3	0.77	0.69
C20:0	0.50	0.43
C20:1n9	-	0.26
C20:5n3	35.36	-
C21:5n3	1.70	-
C22:5n3	4.95	-
C22:6n3	24.31	-
SAFA	7.02	17.26
MUFA	15.21	70.63
PUFA	77.77	12.11
Omega-3	67.08	0.69

Abbreviations: SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FA, fatty acids.

TABLE 4-2. Clinical and demographic characteristics of the study population.

	Omega-3 (n=31)	Placebo (n=32)	P value
Demographics			
Age (years)	59±13	58±13	.845
Ethnicity			
African American (n, %)	30 (97)	32 (100)	-
Caucasian (n, %)	1 (3)	0 (0)	-
Gender (males, %)	20 (62.5)	12 (38.7)	.059
Income ($ per month)	1015±576	1148±761	.552
Clinical			
Time on dialysis (years)	3.6±2.9	3.3±3.8	.784
BMI (kg/m^2)	28.4±8.3	26.9±7.8	.468
Diabetes mellitus (n, %)	20 (62.5)	20 (64.5)	.868
Kt/v	1.58±0.29	1.63±0.32	.536
Statin usage (n, %)	11(35.5)	9 (28.1)	.530
C-reactive protein (mg/dL)	5.8±9.2	4.9±5.6	.134

Abbreviations: BMI, body mass index; Kt/v, index of dialysis adequacy.

Notes: Differences in age, time on dialysis, BMI, Kt/v and income were tested using independent t-test. Baseline CRP levels is reported in median±interquartile range. Differences were tested using Mann-Whitney rank-sum test. Differences in gender, Diabetes mellitus and statin treatment were tested using chi-square. P<0.05 is considered significant.

TABLE 4-3. Means and standard deviations of lipid profiles (TC, HDLC, LDLC, and TAG) at baseline and month-6.

	Placebo(n=26)				Omega 3 (n=28)				P overall
	Baseline	Month 6	**Diff.**	P	Baseline	Month 6	**Diff.**	P	
Lipid profiles									
TC (mg/dL)	159±49	138±42	**-21±26**	.000	176±36	139±29	**-37±32**	.000	**.057**
HDLC (mg/dL)	44±13	49±11	**+5±8**	.004	41±14	47±16	**+6±8**	.000	**.531**
LDLC (mg/dL)	94±42	69±35	**-25±27**	.000	108±34	71±29	**-39±33**	.000	**.092**
TAG (mg/dL)	104±69	96±68	**-7±61**	.561	123±60	102±53	**-20±55**	.064	**.405**
LDL-HDL ratio	2.2±1.0	1.5±0.9	**-0.7±0.8**	.000	3.0±1.6	1.7±1.0	**-1.3±1.1**	.000	**.043**

Abbreviations: TC, total cholesterol; HDLC, high density lipoprotein cholesterol; LDLC, low density lipoprotein cholesterol; TAG, triacylglycerides; Diff, Mean difference of month-6 minus baseline.

Notes: All values are presented as mean±SD. P values for each group derived from paired t-test between baseline versus month-6 comparison. Overall P values derived using independent t-test, tested for mean differences of lipid profiles parameters between omega-3 and placebo groups (bold face).

Data excludes values from 7 subjects (Placebo, n=4; Omega-3, n=3) who passed away (n=4, 2 from each group) or were hospitalized (n=3), during the course of the study. One subject (placebo) was excluded due to extremely high TAG levels (478mg/dL) as the Friedwald equation is not reliable when TAG levels exceed 400mg/dL.

TABLE 4-4. Means and standard deviations of nutritional status indicators (Ser. Alb, Hgb, MIS score, nPNA and BMI) at baseline and month-6.

	Placebo (n=27)				Omega-3 (n=28)				P overall
	Baseline	Month 6	**Diff.**	P	Baseline	Month 6	**Diff.**	P	
Nutritional status									
Ser. Alb (g/dL)	3.7±0.2	3.8±0.4	**0.0±0.3**	.756	3.6±0.3	3.7±0.3	**0.1±0.3**	.070	**.295**
Hgb (g/L)	11.0±1.0	11.3±1.7	**0.2±1.7**	.471	10.4±1.1	10.8±1.5	**0.3±1.4**	.282	**.866**
MIS score	7.6±3.6	8.1±4.0	**0.6±4.0**	.215	9.0±3.6	9.1±3.4	**0.4±2.2**	.408	**.730**
nPNA	0.96±0.38	0.91±0.22	**-0.01±0.23**	.810	0.99±0.29	0.87±0.25	**-0.07±0.45**	.471	**.598**
BMI (kg/m^2)	25.4±5.6	25.1±6.1	**0.3±1.8**	.327	28.1±7.0	28.1±7.2	**0.1±1.0**	.699	**.500**

Abbreviations: Ser.Alb, serum albumin; Hgb, hemoglobin; MIS, malnutrition inflammation score; nPNA, normalized protein equivalence of nitrogen appearance; Diff, mean difference of month-6 minus baseline.

Notes: All values are presented as mean±SD. P values for each group derived from paired t-test between baseline versus month-6 comparison. Overall P values derived using independent t-test, tested for mean differences of lipid profiles parameters between omega-3 and placebo groups (bold face).

Data excludes values from 7 subjects (Placebo, n=4; Omega-3, n=3) who passed away (n=4, 2 from each group) or were hospitalized (n=3), during the course of the study.

TABLE 4-5. Means and standard deviations of inflammatory indicators at baseline and month-6.

	Placebo (n=27)			Omega-3 (n=28)			P overall
	Baseline	Month 6	P	Baseline	Month 6	P	
NFkB (fold diff)	2.48±1.03	2.54±1.14	.703	2.52±1.19	2.15±0.4	.129	**.134**
CRP (mg/dL)*	6.6±8.3	11.0±13.9	.040	13.1±17.5	14.6±19.7	.565	**.259**
CRP (mg/dL)**	4.9±5.6	4.6±12.0	.034	5.8±9.2	6.1±14.0	.412	**.326**

Abbreviations: CRP, C-reactive protein; NFkB, Nuclear factor kappa-light-chain enhancer of activated B cells.

Notes: NFkB value is presented as mean fold difference (relative to control) ±SD. CRP value is presented as *mean±SD and **median±IQR. P values for each group derived from either paired t-test (NFkB)/Wilcoxon rank sum test(CRP) between baseline versus month-6 comparison. Overall P values derived using either independent t-test/Mann-Whitney rank sum test, tested for mean differences of inflammatory parameters between omega-3 and placebo groups (bold face).

Data excludes values from 7 subjects (Placebo, n=4; Omega-3, n=3) who passed away (n=4, 2 from each group) or were hospitalized (n=3), during the course of the study.

FIGURE 4-1. Omega-3 study's flow chart.
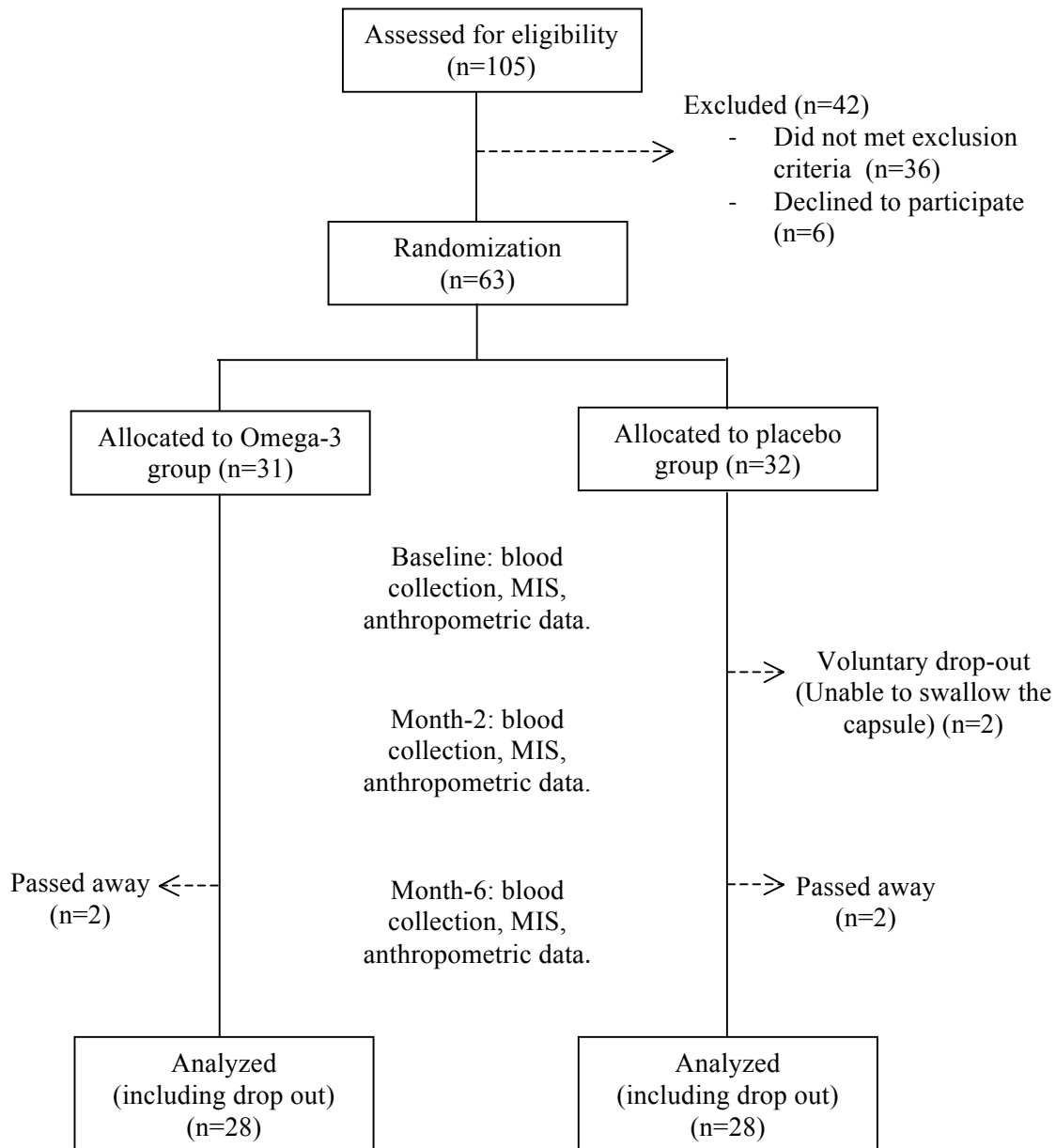
Abbreviations: MIS, Malnutrition-Inflammation Score.

Notes: All patients at the Great Lake Dialysis clinic (n=105) were screened for eligibility. Sixty-nine patients met the inclusion criteria but 6 of them declined to participate. The remaining 63 patients were randomized into omega-3 and placebo groups and underwent intervention for 6 months. Seven additional subjects (Placebo, n=4; Omega-3, n=3) were excluded from data analysis due to death (n=4, 2 from each group) or hospitalization (n=3). Two patients voluntarily dropped out as they were unable to swallow the capsules but their data were included in the analysis (intent to treat).

CHAPTER V

SPECIFIC AIM 2:

EFFECTS OF TOCOTRIENOL-RICH FRACTION SUPPLEMENTATION ON

NUTRITIONAL INDICES, INFLAMMATORY MARKERS, OXIDATIVE STATUS AND

LIPID PROFILES IN CHRONIC HEMODIALYSIS PATIENTS

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Introduction

End-stage renal disease (ESRD) patients on chronic hemodialysis (HD) experience a higher risk

for atherosclerotic vascular disease; cardiac death accounts for approximately 50% of all-cause

mortality [209], which is 15-30 times higher than an age-matched general population [3, 5]. The

increased cardiovascular risk in this setting however cannot be exclusively explained by

traditional cardiovascular risk factors alone. Instead, a combination of various factors especially

malnutrition-inflammation complex syndrome, oxidative stress and dyslipidemia are implicated

[11, 12, 210].

Patients on HD treatment often present with malnutrition-inflammation complex

syndrome characterized by coexistence of protein-energy malnutrition and inflammation

pertaining to dialysis related factors, disease processes, various comorbidities, nutrient loss and

poor nutritional intake [74]. Chronic systemic inflammation which is commonly found in 30-

50% of ESRD patients exacerbates oxidant production. This is aggravated by low intake of

antioxidants, secondary to malnutrition and dietary restrictions in this population [211]. In fact, low circulating plasma levels of antioxidants have been shown to correlate with carotid atherosclerosis in ESRD patients [212]. Equally noteworthy, unadjusted analyses of 4D (Die Deutsche Diabetes Dialyse Studie) study revealed that HD patients in the lowest plasma vitamin E (α-tocopherol) quartile had a 79% higher risk of stroke and 31% higher risk of all-cause mortality as compared to their counterpart in the highest quartile; however, this was confounded by malnutrition [213].

Additionally, in ESRD patients on dialysis, dyslipidemia is highly prevalent due to delayed catabolism of triglyceride-rich apolipoprotein B containing lipoproteins, which subsequently leads to elevated triacylglycerol (TAG) and low plasma HDLC [15]. In a study of a large cohort of incident dialysis patients (n= 21, 893), it has been shown that dyslipidemia present in 82% of the population was predominantly manifest as elevated TAG (52%) and very low density lipoprotein (VLDL) (52%), and decreased HDL (51%) [214].   Furthermore, accumulating evidence indicates that the concentration of plasma apo-A1 and lecithin-cholesterol acyl-transferase (LCAT) are decreased [16, 17] which in turn impedes HDL-mediated reverse cholesterol transport [18], a process of disposing excessive cholesterol from extrahepatic tissues and blood vessel walls. Taken together, a combination of oxidative stress, lipid disorders and inflammation in this population is highly conducive for atherogenesis by promoting LDL oxidation, monocyte activation, endothelial injury and consequently accumulation of lipids in the artery wall [18].

Dietary intervention may be potentially beneficial in improving inflammatory status, correcting oxidative stress and the observed dyslipidemia. However, there is paucity of

information regarding the impact of dietary interventions with nutrients possessing antioxidant, anti-inflammatory or lipid altering properties to correct these problems in the HD population.

Tocotrienols (TT) are dietary compounds that have gained increasing scientific scrutiny. TT are members of the Vitamin E family which also includes Tocopherols (TP). Vitamin E is comprised of 8 different isomers (four TP and four TT) designated α-, β-,γ- and δ, characterized by a different number and position of methyl group attached on the chromanol ring. TT are the unsaturated members of the vitamin E family, found primarily in palm fruit oil, rice bran and annatto. In the US diet however, TP particularly γ-TP is the most abundant form of vitamin E, derived primarily from plant seeds and vegetable oils [117].

Comparisons of antioxidant activities amongst vitamin E family members in rat liver microsomes revealed that TT exhibited 40- to 60- fold higher activities against lipid peroxidation (Fe^{2+} + ascorbate and Fe^{2+} + NADPH induced) and greater radical scavenging potency than TP [129, 130]. However, *in vivo,* antioxidative properties of TT could be limited by physiological process (e.g. absorption, distribution, metabolism and excretion), but TT have been found to be as equally potent as TP [116, 215]. More importantly, TT also play a role beyond their known antioxidant activity – i.e. they possesses an anti-inflammatory property which has been shown to inhibit lipopolysaccharide-induced secretion of interleukin-6 (IL-6) and tumor necrosis factor (TNF) in macrophages [37], and decreased C-reactive protein (CRP) in human volunteers [135]. Additionally, TT may also confer cardioprotective effects by improving lipid profiles (reduced total cholesterol and TAGs) [142, 216], and lower oxidation of LDL cholesterol [137].

To our knowledge, there are no reported TT studies in HD patients, whereas 17 TP trials have been reported, albeit with inconclusive results [24]. Majority of these studies were open

label aimed at improving oxidative stress while only three studies used a randomized controlled trial (RCT) design that included 95 patients cumulatively. Of the three studies, Diepeveen et al[166] provided 800IU/day for 12 weeks (~536mg) of α-TP in combination with 40mg/day of atorvastatin but failed to show any effects on plasma-oxidized LDL. On the other hand, Uzum et al[167] provided 300mg TP/day for 20 weeks and showed decreased plasma oxidative stress markers (MDA and erythrocyte osmotic fragility).

Despite the lack of TT studies in ESRD population, several studies have shown beneficial effects of TT supplementation in the general population. Cardioprotective effects of TT in these studies have been primarily attributed to its anti-oxidative, anti-inflammatory and lipid lowering properties. In a study by Heng et al [135], TT supplementation in healthy volunteers resulted in lower CRP levels, which has been regarded as a prognostic indicator for cardiovascular events and mortality in ESRD patients [6, 7]. In addition, supplementation with TT-rich fraction from palm oil (200mg/day for 8 weeks) plus statin in hypercholesterolemic human subjects resulted in improved lipid profiles while minimizing the side effects of statins [142]. A recent study showed that palm oil derived TRF increased plasma HDL-C in the elderly following 6 month of supplementation [132]. Lipid altering effects of TT was attributed to their ability to suppress, and stimulate ubiquitination and degradation of HMG-CoA reductase as well as to block the processing of SREBPs as demonstrated in cell culture studies [139, 217].

Given that HD patients experience oxidative stress, chronic inflammation and dyslipidemia and that TT are potent antioxidant and anti-inflammatory agents with the potential to improve lipid profiles, we hypothesized that TT supplementation would be of benefit to HD patients by improving one or more of the above conditions. The results from this pilot study form

the basis of the current report. To the best of our knowledge, this is the first study to report the role of TT in HD patients.

Methods

Patients

All patients (n=118) from the Great Lake Dialysis Clinic (Detroit, MI, USA) were screened for eligibility. Patients were eligible for enrolment if they were at least 18 years of age, and had been on dialysis treatment for at least 3 months prior to the study. Patients who were residents of nursing homes or receiving nutritional support (tube feeding/ intradialytic parenteral nutrition) were excluded. None of the patients in the unit were known to have AIDS or to be on active treatment for cancer. All patients were undergoing dialysis with the same high-flux dialyzer membrane (Fresenius Optiflux Dialyzer, Fresenius Medical Care, Waltham, MA, USA).

Study design and procedures

The present study was approved by the Human Investigation Committees of Wayne State University and Great Lake Dialysis, LLC. Written informed consent was obtained from all patients. A total of 88 patients met the inclusion criteria, but, seven patients refused to participate leading to a final number of 81. Based on the final number of participants, randomization was made in blocks in order to keep the sizes of the treatment groups similar and groups were uniformly distributed by key-outcome measures. Block sizes were randomly selected to reduce the potential for selection bias. This process was performed using the Random Allocation Software version 1.0 [218]. A total of 81 patients were randomly allocated into TRF (n=41) and placebo (n=40) groups. All clinical investigators and laboratory personnel as well as the study

participants were blinded from group allocation and treatment until the end of the study. A summary of the study's flowchart is shows in **Figure 5-1**.

Nutritional intervention and compliance

TRF and placebo capsules were formulated by Carotino Sdn. Bhd. (Johor, Malaysia). TRF soft gel capsules used were commercially available, each consisted of 90mg TT (comprising 30.18 mg α-, 5.30 mg β-, 41.66 mg γ-, and 12.86 mg δ-TTs) and 20.00 mg α-TP. Placebo capsules contained negligible amounts of TT (0.12 mg α-, 0.06 mg β-, and 0.06 mg γ-TT) and TP (0.29 mg α-, 0.04 mg β-, and 0.11 mg γ-TP-) (**Table 5-1**). Patients were provided with two TRF or placebo capsules during each of three weekly dialysis sessions. They were also supplied with a pill organizer that contained TRF or placebo capsules for non-dialysis days. Patients were instructed to consume two capsules during main meals (one during lunch and one during dinner). Compliance was ascertained by "directly observed treatment" [175] – which involved nurses directly observing patients taking their supplement capsules during designated days of dialysis. Supplementation adherence on the non-dialysis days was measured by pill counting as described elsewhere [219]. Briefly, patients returned the pill organizer each week and received refills - the left over soft capsules in the pill organizer were counted and recorded. Potential changes in diet pattern were ascertained by collecting 24-hour diet recalls from all subjects (at baseline and week 16) by the same Registered Dietitian. Energy, macro- and micronutrients intake were analyzed using Nutritionist-Pro (First Databank, Chicago, IL) based on the USDA database. Because TT were not included in USDA database, their content was estimated based on published values [115, 220, 221].

Anthropometry, blood sampling and laboratory measures

Anthropometry measurements comprised height and weight. Patients' weights were recorded to the nearest 0.1kg (Tronix Flush-Mounted In-Floor Scale, Scale-Tronix, White Plains, NY, USA) after each HD session to determine estimated dry body weight. Height was measured to the nearest 0.1cm using a Tanita Wall Mounted Height Rod (Tanita, Arlington Heights, IL, USA). BMI was calculated based on the Quetelet's Index [170].

Approximately 15mL of fasting (for ~10 hrs) midweek predialysis blood samples were collected from existing access sites of each study participant into two blood collection tubes. Blood samples were collected into tubes (Becton Dickson, Franklin Lakes, NJ, USA) containing either EDTA (for lipid analyses) or lithium heparin (for inflammatory markers and oxidative status analyses) at baseline and wks-8 (lipid analyses only), 12 and 16. Plasma was isolated by centrifugation at 2800rpm for 20 minutes at 4°C, divided into aliquots and immediately stored at -80°C until further analysis. Serum samples for standard renal profiles (serum albumin, BUN, creatinine) were analyzed using standard automated laboratory techniques by an external laboratory (Ascend Clinical Laboratory Services, Redwood City, CA, USA).

Plasma Total cholesterol (TC), and TAG was determined by enzymatic reaction (Pointe Scientific Inc., Canton, MI, USA). HDLC was measured in the supernatant after precipitation of apoB-containing lipoproteins by dextran sulfate and magnesium ions (Pointe Scientific Inc., Canton, MI). LDLC was calculated using the Friedewald equation by difference (LDLC = TC minus HDLC minus TAG/5).

CRP levels were measured using commercial kits based on enzyme-linked immunoabsorbent assay (ELISA) method as per the manufacturer's protocol (Cayman Chemical,

Ann Arbor, MI, USA). Plasma samples were diluted in the assay buffer into 1:15,000 and 100µL of each sample was placed into a 96-well microplate that had been coated with a monoclonal antibody specific for human CRP and incubated for an hour. The plate was then rinsed four times with wash buffer before addition of horseradish peroxidase-labeled CRP monoclonal antibody. Two antibodies were added and formed a sandwich by binding to different locations on the CRP molecule. CRP concentration in the plasma samples were measured at 450nm after addition of chromogenic substrate tetramethybenzidine that formed a distinct yellow color. IL-6 (Thermo Scientific, Rockford, IL, USA) , ApoA1 (Immunology Consultants Lab. Inc., Portland, OR, USA) and TNFα in the plasma was also measured using similar principles per manufacturers' protocol. NFκB p65 levels were measured in the nuclear extract of peripheral blood mononuclear cells as described in details in Chapter III.

Total antioxidant power (TAP) of the plasma was measured using commercial kits based on a cupric reducing antioxidant capacity spectrophotometric method (Oxford Biomedical, Oxford, MI, USA). Briefly, 200µL of diluted plasma samples (1:40 of plasma/dilution buffer) were placed in a 96-well microplate followed by the addition of copper solution (50µL) and stop solution (50µL). The reduction potential in the plasma sample converts Cu^{2+} into a reduced form of copper (Cu^{1+}) which in turn formed a stable 2:1 complex with the chromogenic reagent. This color complex was quantified with an absorption maximum at 450nm. Standard curve was prepared using a known concentration of trolox with data being expressed as mM trolox equivalents.

Malondialdehyde (MDA) in the plasma was measured indirectly based on the reaction of a chromogenic reagent, 2-thiobarbituric acid with MDA using a spectrophotometric method (Oxford Biomedical, Oxford, MI, USA). Plasma samples were deprotinized with trichloroacetic

acid (Fisher Scientific, Fair Lawn, NJ, USA) and the supernatant was obtained by centrifugation. Total MDA in the deprotinized plasma samples were determined by the addition of indicator solution followed by incubation at 65°C for 30 mins and the color complex was quantified with an absorption maximum at 540nm. Standard curve was prepared using a known concentration of MDA stock solution supplied in the kit.

Statistical Analysis

All results were analyzed using intention-to-treat principle in which all data were counted regardless of post-randomization withdrawal and any missing values were reported accordingly. Statistical analyses were carried out using SPSS v.16 (IBM, Chicago, IL, USA). Data for categorical variables were reported as number and percentage, while continuous data were reported as mean±standard deviation or median±interquartile range for skewed variables. Between group difference in the change in parameters at particular time points were tested using independence t-test (or respective non-parametric test for non-normality distributed variables). One way repeated measures ANOVA were used to test time effects (the change from baseline) for variables of interest in each group. χ2 statistics was used to evaluate differences in categorical variables. Correlations between selected variables were calculated using Pearson's correlation coefficients test. P values of <0.05 is regarded as statistically significant.

Results

Characteristics of the study population

A total of 81 patients were randomized into placebo (n=40) and TRF (n=41) groups and were provided either TRF or placebo capsules for 16 weeks. Two patients in the placebo group died

due to cardiac arrest during the course of the study. An additional two patients (1 placebo; 1 TRF) were excluded due to transplantation and catheter dysfunction, respectively.

Table 5-2 shows the clinical and demographics characteristics of the study population. There were no significant differences in all demographic and clinical variables at baseline. Our study population was homogenously comprised of African-American ethnicity. Average body mass index for placebo (28.7±8.2 kg/m^2) and TRF (30.3±8.1 kg/m^2) groups were in the category of overweight based on WHO (2000) classification [171]. Over 60% of the patients in both placebo and TRF groups had diagnosed Diabetes Mellitus. Moreover, ten patients (5 placebo; 5 TRF) was positive for hepatitis C antibody test (RIBA Reflex Siemens Centaur chemiluminescent assay). Lipid lowering drugs (statins) were used by 28 patients (11 in placebo; 17 in TRF), aspirin were prescribed to 47 patients (22 placebo; 25 TRF) and 69 patient were prescribed one or more type of antihypertensive drugs (35 placebo; 34 TRF). However, there were no differences in term of drug distribution between the two groups based on χ^2 test. In term of dialysis parameters, both placebo and TRF groups had comparable dialysis treatment adequacy as measured by Kt/V (K – dialyzer clearance of urea, t- dialysis time, and V- volume of distribution of urea). In term of vascular access route, both groups had a similar proportion of patients using either an arteriovenous fistula (18 placebo; 15 TRF), an arteriovenous graft (13 placebo; 16 TRF) or venous catheter (9 placebo, 10 TRF).

Dietary intake, tolerability, side effects and compliance

Dietary analysis based on 24-hr dietary recall during baseline and wk-16 of the study showed that there were no changes in energy and macronutrients, between the two groups at a given particular time point. There were no differences in vitamin E (TP and TT) intake at baseline and

wk-16. Similar results were also seen in vitamin C, β-carotene and other micronutrients (potassium, phosphorus) intake within and between the groups (**Table 5-3**). Meanwhile, a total of 62% of our study population [58% placebo (n=23) and 66% TRF (n=27)] were taking dietary supplements that comprised of Renalcaps (18% placebo; 18% TRF), Nephrocaps (3% placebo; 3% TRF), vitamin C (0% placebo; 2% TRF) and multivitamins (2% placebo; 2% TRF). Nephrocaps and Renalcaps comprised of 100mg vitamin C, 1.5mg Thiamine, 1.7mg Riboflavin, 20mg Niacin, 10mg Vitamin B6, 1 mg Folate, 6μg vitamin B12, 150μg Biotin and 5mg pantothenic acid (**Figure 5-2**).

TRF and placebo supplements were well tolerated by the subjects. During the first week of the study, one patient from the placebo group reported of diarrhea but this was not related to supplementation and subsequently resumed the study. No other adverse effects were reported from subjects in either groups to justify removal from the study. In the current study, compliance was ascertained by direct and real-time observation of subjects taking the supplements during dialysis sessions (three times a week) as well as pill counting method on non-dialysis days. Compliance during non-dialysis days was more than 70% for each week except during wk-14 and wk-15 due to holidays (Thanksgiving). There were no differences in capsule consumption (% of capsule disappearance) between placebo and TRF groups during the course of study (**Figure 5-3**). Compliance of supplementation at home was relatively good considering that the study population was comprised of low income and low literacy group which typically is associated with poor compliance [175, 222].

Effects on nutritional, inflammatory and oxidative markers

Nutritional, inflammatory and oxidative indices are presented in **Table 5-4.** Data for CRP and IL-6 are reported in median±IQR because they were skewed. Median CRP levels at baseline was 16.6±28.8 and 13.0±20.5mg/L for the placebo and TRF group, respectively. There were no difference in CRP levels between TRF and placebo at each time point. No significant changes were noted in the CRP levels in both groups per repeated measures one-way ANOVA. Similarly, there was no difference in mean IL-6 between or within groups at all time points.

Similarly, there were no significant changes (repeated measures one-way ANOVA) observed in NFκB p65 levels over the time in both groups. It is important to note that, at baseline, NFκB p65 levels in TRF group is significantly higher than placebo (91.7±9.8 *vs* 54.2±10.5 pg/μL, p<0.05, respectively). Upon normalization into the baseline value (week-12 minus baseline), TRF group showed marginal difference (p=0.08) when compared to placebo (-16.7±13 *vs* 15.1±11 pg/μL) (**Figure 5-4**). Another inflammatory marker, TNFα, also demonstrated similar results in which there were no significant difference noted within and between groups as measured at baseline and week-12 of the intervention (**Figure 5-5**).

Measurement of TAP showed that the TRF group had significantly higher TAP (p<0.05) at wk-12 as compared to placebo (626±98 *vs* 564±95 mM Trolox equivalent). However, no changes were observed within the TRF and placebo groups when compared to the baseline. This was also in concert with indirect measurement of MDA, a marker for oxidative stress, in which TRF group had a marginally lower MDA (2.60±2.28 μM MDA) at week-12 as compared to placebo group (4.68±5.72 μM MDA) (p=0.055). There were no significant changes in MDA values within the TRF and placebo groups when compared to the baseline. In terms of nutritional

indicators (serum albumin, hemoglobin, BMI), no changes were observed within or between groups.

Effects on lipid profiles

Plasma TAG levels was significantly reduced in TRF group after 12 weeks of supplementation as compared to baseline values (144±91 *vs* 113±47 mg/dL plasma, p<0.05) and remained significantly reduced at wk-16 (144±91 *vs* 103±45 mg/dL plasma, p<0.05). In contrast, TAG levels remained the same in the placebo group. Interestingly, both groups showed a progressive decline in plasma TC and a significant improvement in HDLC when compared to the baseline values starting at wk-8. Hence, this also resulted in a reduction of LDL-C and the ratio of TC/HDLC (**Table 5-5**).

As TAG levels were somewhat higher in the TRF group at baseline, values were normalized to these baseline values. Normalized plasma TAG were reduced in the TRF group as compared to placebo at wk-12 (-33±84 vs 6±66 mg/dL, p=0.032), and at wk-16 (-36±79 vs -8±47 mg/dL, p=0.072) (**Figure 5-6**). Plasma HDLC was significantly higher in the TRF group as compared to placebo at both wk-12 (22±15 vs 9±11 mg/dL, p<0.0001) and wk-16 (16±14 vs 10±9 mg/dL, p<0.05, respectively) (**Figure 5-7**).

Measurement of ApoA1 concentration in the plasma, a major protein component of HDL particles, revealed that it was significantly higher in TRF group as compared to placebo at wk-12 (1.56±0.59 vs 1.27±0.34 mg/mL, p<0.05, respectively) consistent with the higher HDL-C concentrations (**Figure 5-10**). However, no difference was noted between groups at wk-16.

Measurement of CETP activity, a plasma protein that facilitates the transfer of cholesteryl ester from HDL in exchange for triacylglycerols from TAG-rich lipoprotein, was significantly

lower in the TRF group at wk-16 as compared to placebo (96±18 vs 129±43 pmol/mL plasma/hr, p<0.001, respectively), which reflected the higher HDL-C value in TRF group. Interestingly, CETP activity was slightly higher in TRF group during wk-12 (95±19 vs 84±19 pmol/mL plasma/hr, p<0.05, respectively). Pearson's correlation test between HDL-C levels and ApoA1 concentration during wk-12 and wk-16 showed a significant correlation between the two variables. Similarly, significant correlations were observed between plasma TAG and CETP (**Figure 5-11**).

Correlation between biochemical parameters

Table 5-6 depicted Pearson's correlation coefficient test between nutritional indicators, inflammatory markers and oxidative status parameters based on pool data (baseline + week-12 + week-16). MDA as indicator of oxidative stress has an inverse relationship with TAP (r= -0.273, p<0.01) indicating that higher total antioxidant capacity in the plasma confers some protective effect against the reactive species – MDA. Moreover, MDA levels were also significantly correlated with CRP (r=0.501, p<0.01), exemplifies the complex relationship of inflammation and oxidative stress as discussed in Chapter II. Serum albumin was also positively correlated with TAP suggesting the antioxidant role of albumin (by localized thiol moieties) as previously reported elsewhere [11]. Furthermore, serum albumin was inversely correlated with CRP levels (r= -0.163, p<0.05) due to the fact that it is a negative-acute phase protein while CRP is the positive-acute phase protein. Increased in CRP is therefore lead to a decreased in serum albumin.

The summary of Pearson's correlation coefficient test between lipid profiles (TC, TAG, HDLC, LDLC and TC-HDLC ratios) is presented in **Table 5-7**. In brief, CRP was positively correlated with TAG (r= 0.159, p<0.05) while had an inverse relationship with HDLC (p= -

0.163, p<0.05) suggesting the protective role of HDLC. Similarly, LDLC showed a positive relationship with MDA (r= 0.138, p<0.05). Interestingly, when compared to dietary intake parameters, total fat and cholesterol intake were inversely correlated with HDLC, while cholesterol and vitamin E intake had a positive relationship with TAG levels.

Discussion

The present study, to the best of our knowledge, is the first to report the extent of vitamin E tocotrienol-rich fraction supplementation effects on inflammatory markers, oxidative status and lipid profiles in HD subjects. This study highlights some important findings especially in the lipid altering properties of TT which could potentially have clinical implications. Patients in both placebo and TRF group had comparable demographics, percentage of smoking cigarettes, dialysis and clinical parameters distribution. In addition, baseline inflammatory markers, oxidative status and lipid profiles in both groups were not statistically different. Thus any potential comorbidities and confounding factors were minimized by being randomly distributed across the two groups.

In this study, we observed that baseline median CRP levels in both groups were relatively higher as compared to commonly reported values in the literatures [223, 224]. However, this is in accordance with our previous results [175] despite methodological differences between the two studies. The fact that gradual increments in CRP levels were seen in both groups might suggest a progressive inflammatory burden in this population. Results from the present study indicated that short term TRF administration was unable to improve CRP levels in HD patients. This is in contrast with recent TRF trial that has been conducted in healthy volunteers which showed reduction in CRP levels in older subjects [135]. We speculate that this is partially due to high

interindividual variability in CRP levels, which is often associated with transient intercurrent clinical events and the dynamic response of immune system [225]. Despite the high predictive power of mortality in a single CRP measurement [73], given the fact that CRP fluctuates dramatically upon acute inflammation [226] or even in the absence of changes in other biochemical measures or health status [226-228], CRP measurement alone may not adequately capture immunomodulatory changes following therapeutic intervention, thus necessitating measurement of other inflammatory biomarkers. Similar observations were also seen in IL-6, NFκB p65 and TNFα values. In term of oxidative status parameters, no significant improvements were seen in TAP and MDA values in either TRF or placebo group over time, but the TRF group had higher TAP and marginally lower MDA at week-12 but these was not seen at week-16. Taken together, TRF supplementation failed to improve inflammatory and oxidative status markers in the current study.

Interestingly, TRF supplementation resulted in improvement in plasma TAG and HDLC. The improvement in lipid profiles was less anticipated due to mixed results in previous human trials. For example, Mustad et al [144] administered 200mg TT/day for 28 days in healthy hypercholestrolemic volunteers but failed to show any effects on blood lipids. Similarly, supplementation with a TT-rich vitamin E concentrate (140mg TT, 80mg TP per day) to 20 men for 42 days showed no improvement in lipoprotein profiles despite changes in plasma tocopherols and tocotrienols concentration [143]. We speculate that the earlier studies in human volunteer did not show a positive result in blood lipids due to a shorter duration, a smaller dose and sample size. A recent study which administered TT for a longer period (6 months) in healthy older adults showed significant improvement in lipid profiles (increased HDL-C and improved

TC/HDLC ratios) [132], which strengthen the speculation that TT effects on blood lipids could be a function of time and may depend on subject's clinical status.

Because dyslipidemia is implicated in the pathogenesis of cardiovascular diseases, therefore, it has become more common for dialysis patients with dyslipidemia to receive statin treatment [229], albeit with conflicting evidence [69]. In our study population, 11 patients in placebo group and 17 patients in TRF group were on statin treatment. Statin induced inhibition of HMG CoA reductase leads to depletion of hepatic cholesterol pools and subsequently decreases production of apoB-containing VLDL particles and upregulates LDL receptors. Because VLDLs are the major TAG-trafficking lipoprotein in fasting plasma, therefore statins reduce TAG by enhancing clearance of VLDL and reuptake of LDL via LDL receptors. In the present study, TAG levels were significantly reduced in TRF groups at wk-12 but not at wk-16 (p=0.07) partly due to high variability in both groups. We postulated that this variability may partly be due to statin users in the placebo group. Additional analysis among statin users revealed that the TAG reduction observed in the placebo group was indeed due to statin administration (**Figure 5-8**). Interestingly, in TRF group, both the statin and non-statin users showed a reduction trend in TAG, which strengthens the speculation that TRF may be directly involved in the TAG reduction process. Similar findings has been shown in a randomized, placebo-controlled clinical trial by Zaiden et al [140] in which supplementation with 120mg of γ-,δ-tocotrienols (γ-,δ-T3) for 8 weeks resulted in an ~ 28% reduction in TAG as compared to baseline. This improvement in TAG was partly explained by suppression of the upstream regulators of lipid homeostasis as demonstrated in an in-vitro study [140].

The increase in HDL-C levels in the plasma can be attributed in part to increased production of HDL particles, delayed catabolism of HDL particles or higher cholesterol contents

in HDL particles. To elucidate the mechanism of TT effects on TAG and HDL-C during wk-12 and wk-16, ApoA1 and CETP were measured. Our results showed that the ApoA1 level was significantly higher in the TRF group during wk-12, which coincided with higher HDL-C in the TRF group. Because ApoA1 is the major protein component of HDL particles (70%), increased HDL-C levels at wk-12 in the TRF group may be explained by an increase in HDL particles (mirrored by higher ApoA1 concentration). Pearson's correlation test between HDL-C levels and ApoA1 concentration during wk-12 and wk-16 confirmed significant correlation between the two variables.

Animal studies have established that about one third of ApoA1 is catabolized by the kidney and the rest by the liver - humans are assumed to have similar pathway of ApoA1 catabolism [230, 231]. In the context of ESRD patients, because kidney functions are impaired, this also hypothetically leads to reduction of ApoA1 catabolism. Conversely, a recent study showed that ESRD patients on HD have higher prevalence of autoantibodies to ApoA1 (anti-ApoA1 IgG) which correlates with dialysis vintage [232]. Similarly, in a comparative study between healthy control and hemodialysis patients, Pahl et al [233] found that plasma HDL-C is markedly reduced in HD patients which was mirrored by reduced ApoA1 plasma concentration. Taken together, these may partially explain low HDL-C observed in ESRD/HD patients.

In the context of our study, the TRF supplemented group had significant increase in HDL-C which was reflected by higher plasma ApoA1 concentration. In concert with the current study, Heng et al [135] demonstrated that supplementation with 150mg/day TRF in a healthy population resulted in increased expression of ApoA1 precursor. The effect on ApoA1 may not be unique to TT per se because α-TP supplementation also increased pro apoA1 and apoA1 concentration [234]. Literature on molecular explanation of TRF effects on ApoA1 expression is

scarce, thus further investigation in this area is warranted. It is possible that TRF and tocopherol exhibit antioxidant like mechanism which implicates several transcription factors such as MAP, PPARα and PPARγ in the upregulation of proapoA1 [234].

CETP activity was lower in TRF group during wk-16, consistent with the higher HDLC value. CETP mediates the transfer of cholesterol ester from HDL to VLDL remnants in exchange for triacylglycerols [18]. Because HD patients usually experience delayed catabolism of TAG rich lipoprotein, therefore, we speculate that CETP activity may be increased to facilitate TAG-Cholesterol transfer between HDL and apoB100 lipoproteins. A recent study that compared HDL-associated enzymes in HD *vs* healthy control subjects confirmed that HD patients have higher CETP as compared to control [71], while another study reported no changes in CETP [18]. In our study Pearson's correlation test between CETP activity and TAG concentration during wk-12 and wk-16 confirmed significant correlation between the two variables.

Hemodialysis patients are frequently hospitalized due to concurrent comorbidities. In the current study, we monitored missed dialysis sessions for each patient and the primary reason was recorded. Cumulative numbers of missed dialysis session for each group were calculated (**Figure 5-12**). The total days of hospitalization was 122 *vs* 180 days for the TRF and placebo groups, respectively. This is an intriguing observation. It should be noted that in a recent study in end stage liver disease (ESLD) patients Patel et al reported that supplementation with 400mg TT/day in ESLD patients slowed the rise in MELD scores (a standardized scoring system that is clinically used to determine severity of liver failure and priority for transplantation) in 50% of the patients receiving TT supplementation [169]. Coupled with our initial observation, it is possible that TT therapeutics may provide benefit due to their ability to penetrate vital organ

tissues. However, this hypothesis is purely speculative at present and warrants further investigation in a larger cohort.

It is important to emphasize that the comparisons here are made to the studies that either used α-TP in HD patients or studies that used TT/TRF in healthy volunteers of different clinical population. Given that TT possesses the distinct functions from TP in the context of its antioxidant and anti-inflammatory properties; therefore there are no comparable vis-a-vis studies in HD patients.

Limitations of the study

The present study highlights beneficial treatment effects of TRF on lipids. Nevertheless, the results of this study should be interpreted with caution. Firstly, the study was homogenously comprised of a single race, and the majority of the patients were in low-income, low-literacy group. Therefore, the positive outcome of TRF on lipids may not be generalizable to a more diverse HD population. Secondly, compliance may have been a factor. However our study design enabled us to directly monitor and observe patients taking their supplement during regular dialysis days, while we were able to estimate consumption on non-dialysis days by the pill counting method. Dietary changes were monitored by a single 24-hr dietary recall at baseline and at the end of the study. We acknowledge the limitation of this method to adequately capture dietary changes, however given the fact that diet monotony of dialysis patients plus limited contribution of TT-rich food sources to our patients' diet, we believe that variation in dietary contribution of TT is clinically less important in contributing to the outcome of the present study. Finally, the number of subjects in our cohort did not allow us to separate the effects of the

various medication regimens (e.g. statins, anti-hypertensive drugs, aspirin) from the effects of TT *per se*.

We did not measure TT in the blood, as the focus of the study was to *observe* the global effects following oral supplementation, and not to look at the pharmacokinetics of tocotrienol supplementation *per se*. It is known that TT are transported in circulating lipoproteins,[124, 235, 236] albeit with a short half-life[123] but recent data shows that they are indeed delivered to vital organs.[169] Correlating plasma TT following supplementation in HD patients will be of importance in the future as dose-escalation studies are contemplated to ascertain the optimal dose of TT required in this population.

Tables and figures

TABLE 5-1. TRF and placebo capsule composition

Content (mg)	TRF	Placebo
Tocopherol (TP)		
α-TP	20.00	0.29
β-TP	-	0.04
δ-TP	-	-
γ-TP	-	0.11
Tocotrienol (TT)		
α-TT	30.18	0.12
β-TT	5.30	0.06
δ-TT	41.66	-
γ-TT	12.86	0.06

Notes: TRF capsules were formulated from oil palm fruits while placebo capsules from wheat germ oil. Other ingredients include the same amount of medium chain triglycerides in both capsules.

TABLE 5-2. Baseline clinical and demographic characteristics of the study population.

	Placebo (n=40)	TRF (n=41)	P value
Demographics			
Age (yrs)	58±13	59±12	0.565
Age > 65 yrs (n, %)	12 (30)	14 (34)	0.689
Ethnicity			
African American (n, %)	40 (100)	40 (97.5)	-
Gender (males, %)	23 (57.5)	20 (48.8)	0.432
Smoking cigarettes (n, %)	10 (25.0)	10 (24.3)	
Clinical			
Diabetes Mellitus (n, %)	26 (65)	25 (61)	0.708
Hepatitis C (n, %)	5 (12.5)	5 (12.2)	0.967
Time on dialysis (months)	37±18	34±19	0.446
BMI (kg/m^2)	28.7±8.2	30.3±8.1	0.768
Systolic B/P (mmHg)	150±24	153±23	0.651
Diastolic B/P (mmHg)	81±15	83±14	0.506
Kt/v	1.48±0.26	1.45±0.20	0.516
Statins (n, %)	11 (27.5)	17 (41.4)	0.124
Aspirin (n, %)	22 (55.0)	25 (61.0)	0.297
Anti-hypertensive, one or more type (n,%)	35 (87.5)	34 (82.9)	0.562
Vascular access			0.732
Arteriovenous fistula (n, %)	18 (45)	15 (36.6)	
Arteriovenous graft (n, %)	13 (32.5)	16 (39)	
Venous catheter (n, %)	9 (22.5)	10 (24.4)	

Abbreviations: BMI= Body Mass Index; B/P=blood pressure; Kt/V=Index of Dialysis Adequacy.

Note: Data are reported as mean±SD for continuous variables and number (percentage) for categorical variables. P values are comparison between groups at baseline obtained from independent t-test or Pearson χ2 test wherever appropriate,

TABLE 5-3. Energy, macronutrients and micronutrients intake during the course of the study.

	Baseline				Week-16			
	All (n=81)	Placebo (n=40)	TRF (n=41)	P value	All (n=71)	Placebo (n=34)	TRF (n=37)	P value
Energy (kcal/day)	1995±977	2132±1243	1868±633	N.S.	1544±580	1505±545	1580±616	N.S>
Protein (gm/day)	94±59	99±80	88±31	N.S.	75±40	75±36	76±44	N.S.
Carbohydrate (gm/day)	209±106	204±100	213±111	N.S.	178±79	177±71	180±85	N.S.
Fat (gm/day)	90±68	105±90	76±37	N.S.	60±32	57±23	63±39	N.S.
Vit A (IU/day)	7371 ± 19372	5524 ± 7415	9172 ± 26273	N.S.	6015 ± 9645	6377 ± 12115	5718 ± 7193	N.S.
Vit C (mg/day)	98±84	89±82	108±86	N.S.	90±105	102±112	80±98	N.S.
Vit E (mg/day)	9±10	9±9	10±12	N.S.	6±4	5±3	6±5	N.S.
Tocotrienols (mg/day)	2.5±3.8	2.3±3.8	2.6±3.8	N.S.	1.6±2.3	1.6±1.5	1.6±2.8	N.S.
Potassium (mg/day)	2350±872	2329±1266	2371±2334	N.S.	2015±919	1962±961	2058±894	N.S.
Phosphorus (mg/day)	1183±652	1181±722	1186±585	N.S.	996±469	953±431	1031±500	N.S.

Abbreviations: N.S., not significant

Note: Energy, macronutrients and micronutrients intake were estimated based on 24-hr dietary recall. Reported tocotrienols values are from diet only and does not include contribution from supplementation. All values are presented as mean±SD. P values derived using independent t-test, tested for mean differences of micronutrients intake between TRF and Placebo groups between baseline and wk-16.

TABLE 5-4. Inflammatory, oxidative and nutritional markers during baseline, week-12 and week-16.

	Placebo (n=38)	TRF (n=40)
Inflammatory markers		
C-reactive protein (CRP) (mg/L)		
Baseline	16.6±28.8	13.0±20.5
Week-12	25.1±36.5	15.5±18.0
Week-16	17.9±39.5	14.3±28.0
Interleukin-6 (IL-6) (pg/mL)		
Baseline	4.6±5.9	4.9±3.5
Week-12	5.2±3.5	4.9±2.3
Week-16	5.2±2.1	5.9±3.1
Oxidative Status		
TAP (mM Trolox equivalent)		
Baseline	640±207	669±127
Week-12	564±95[a]	626±98[b]
Week-16	697±166	759±246
TBARS (µM MDA)		
Baseline	3.39±5.12	3.01±4.65
Week-12	4.68±5.72	2.60±2.28
Week-16	3.85±3.90	2.89±3.65
Nutritional indicators		
Serum albumin (g/dL)		
Baseline	3.9±0.3	3.9±0.3
Week-12	3.9±0.3	3.9±0.3
Week-16	4.0±0.4	3.9±0.5
Hemoglobin (g/L)		
Baseline	10±2	10±2
Week-12	10±1	10±2
Week-16	10±1	10±2
Body Mass Index (kg/m^2)		
Baseline	28.7±8.2	30.3±8.1
Week-12	29.1±8.1	30.4±8.2
Week-16	29.1±8.3	30.5±8.2

Abbreviations: TAP,total antioxidant power; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

Note: Values are reported as median±IQR (CRP, IL-6) or mean±SD (TAP, TBARS, Alb, Hb, BMI). Statistical analysis were conducted using one-way repeated measures ANOVA to test the difference in the parameters overtime as compared to the baseline in each group – significant difference ($p<0.05$) for this test denoted as *symbol. Independent t-test (TAP, TBARS, Alb, Hb, BMI) and Mann-Whitney U test (CRP, IL-6) were used to test the mean differences between TRF and placebo groups at particular time point – significant difference ($p<0.05$) for these tests denoted as different alphabetical letters. At week-12, there was marginal difference ($p=0.055$) in TBARS (µM MDA) between placebo and TRF groups.

TABLE 5-5. Lipid profiles of the subjects at baseline, week-8, week-12 and week-16.

	Placebo (n=38)	TRF (n=40)
Triacylglycerols (mg/dL)		
Baseline	109±63	144±91
Week-8	106±51	139±86
Week-12	100±57	113±47*
Week-16	95±48	103±45*
Total cholesterol (mg/dL)		
Baseline	179±42	183±49
Week-8	153±32*	158±36*
Week-12	140±31*	142±43*
Week-16	149±38*	145±45*
High-density lipoprotein cholesterol (mg/dL)		
Baseline	44±12	42±13
Week-8	51±14*	51±15*
Week-12	54±13*[a]	63±18*[b]
Week-16	54±12*	58±18*
Low-density lipoprotein cholesterol (mg/dL)		
Baseline	112±38	112±46
Week-8	81±31*	79±35*
Week-12	70±32*	58±38*
Week-16	75±34*	66±42*
TC/HDLC ratio		
Baseline	4.25±1.36	4.59±1.80
Week-8	3.19±1.08	3.29±1.07
Week-12	2.80±1.13	2.38±0.87
Week-16	2.88±1.07	2.66±1.03

Note: Values are reported as mean±SD. TAG, TC and HDLC were measured using standard enzymatic assay, whilst LDLC were calculated by different using Fridewald equation. One-way repeated measures ANOVA was used to test the difference in the parameters overtime as compared to the baseline in each group – significant difference ($p<0.05$) for this test denoted as *symbol. Independent t-test was used to test the mean differences between TRF and Placebo groups at particular time point – significant difference ($p<0.05$) for these tests denoted as different alphabetical letters.

TABLE 5-6. Pearson correlation coefficient between selected nutritional, inflammatory and oxidative status markers based on pool data (baseline + week 12 + week-16).

	Oxidative Status		Inflammatory Markers				Nutritional Indicators		
	TAP	MDA	IL-6	CRP	TNFα	NFκB	Alb	Hgb	Ferr
TAP	1								
MDA	**-.273****	1							
IL-6	n.s.	n.s.	1						
CRP	n.s.	**.501****	**.148***	1					
TNFα	n.s.	n.s.	n.s.	n.s.	1				
NFκB	n.s.	n.s.	n.s.	n.s.	n.s.	1			
Alb	**.144***	n.s.	n.s.	**-.163***	n.s.	n.s.	1		
Hgb	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	
Ferr	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**.190***	**-.168***	1

Abbreviations: TAP, total antioxidant power; MDA, malondialdehyde; IL-6, interleukin-6; CRP, C-reactive protein; TNFα, tumor necrosis factor alpha; NFκB, nuclear factor kappa B; Alb, serum albumin; Hgb, hemoglobin; Ferr, ferritin; n.s., not significant.

Notes: This figure shows Pearson correlation coefficients of selected nutritional, inflammatory and oxidative status markers based on pool data (n=155 for TNFα and NFκB; n=226 for all other). Data are presented as Pearson correlation *r* values. Symbol * and ** indicates correlation is significant ($p < 0.05$ and $p < 0.01$, respectively).

TABLE 5-7. Pearson correlation coefficient between lipid profiles and selected nutritional, inflammatory and oxidative status markers based on pool data (baseline + week 12 + week-16).

	Lipid Profiles				
	TC	TAG	HDLC	LDLC	TC/HDLC
Oxidative Status					
TAP	n.s.	**.146***	n.s.	n.s.	n.s.
MDA	**.148***	n.s.	n.s.	**.138***	n.s.
Inflammatory Markers					
CRP	n.s.	**.159***	**-.163***	n.s.	n.s.
IL-6	n.s.	n.s.	n.s.	n.s.	n.s.
TNFα	n.s.	n.s.	n.s.	n.s.	n.s.
NFκB	n.s.	n.s.	n.s.	n.s.	n.s.
Nutritional Indices					
Alb	n.s.	n.s.	n.s.	n.s.	n.s.
Hgb	n.s.	n.s.	n.s.	n.s.	n.s.
Ferr	n.s.	n.s.	n.s.	n.s.	n.s.
Calorie/kg body weight	n.s.	n.s.	n.s.	n.s.	n.s.
Protein/kg body weight	n.s.	n.s.	n.s.	n.s.	n.s.
Fat intake (g)	n.s.	n.s.	**-.194***	n.s.	n.s.
Cholesterol intake (mg)	n.s.	**.306***	**-.208***	n.s.	**.183***
Vitamin E intake (mg/day)	n.s.	**.226****	n.s.	n.s.	n.s.

Abbreviations: TAP, total antioxidant power; MDA, malondialdehyde; IL-6, interleukin-6; CRP, C-reactive protein; TNFα, tumor necrosis factor alpha; NFκB, nuclear factor kappa B; ALB, serum albumin; HGB, hemoglobin; FERR, ferritin; n.s., not significant; TC, total cholesterol; TAG, triacylglycerol; HDLC, high density lipoprotein cholesterol; LDLC, low density lipoprotein cholesterol.

Notes: This figure shows Pearson correlation coefficients between lipid profiles and selected nutritional, inflammatory and oxidative status markers based on pool data (n=155 for TNFα, NFκB and dietary data; n=226 for all other). Data are presented as Pearson correlation *r* values. TC, TAG and HDLC were measured in the plasma using commercial kit while LDLC were calculated by difference using Friedewald formula. Dietary data (calorie/kg body weight, protein/kg body weight, fat, cholesterol and vitamin E intake were estimated based on two 24-hr diet recall (baseline and wk-16). Symbol * and ** indicates correlation is significant ($p<0.05$ and $p<0.01$, respectively).

FIGURE 5-1. TRF study's flow chart.
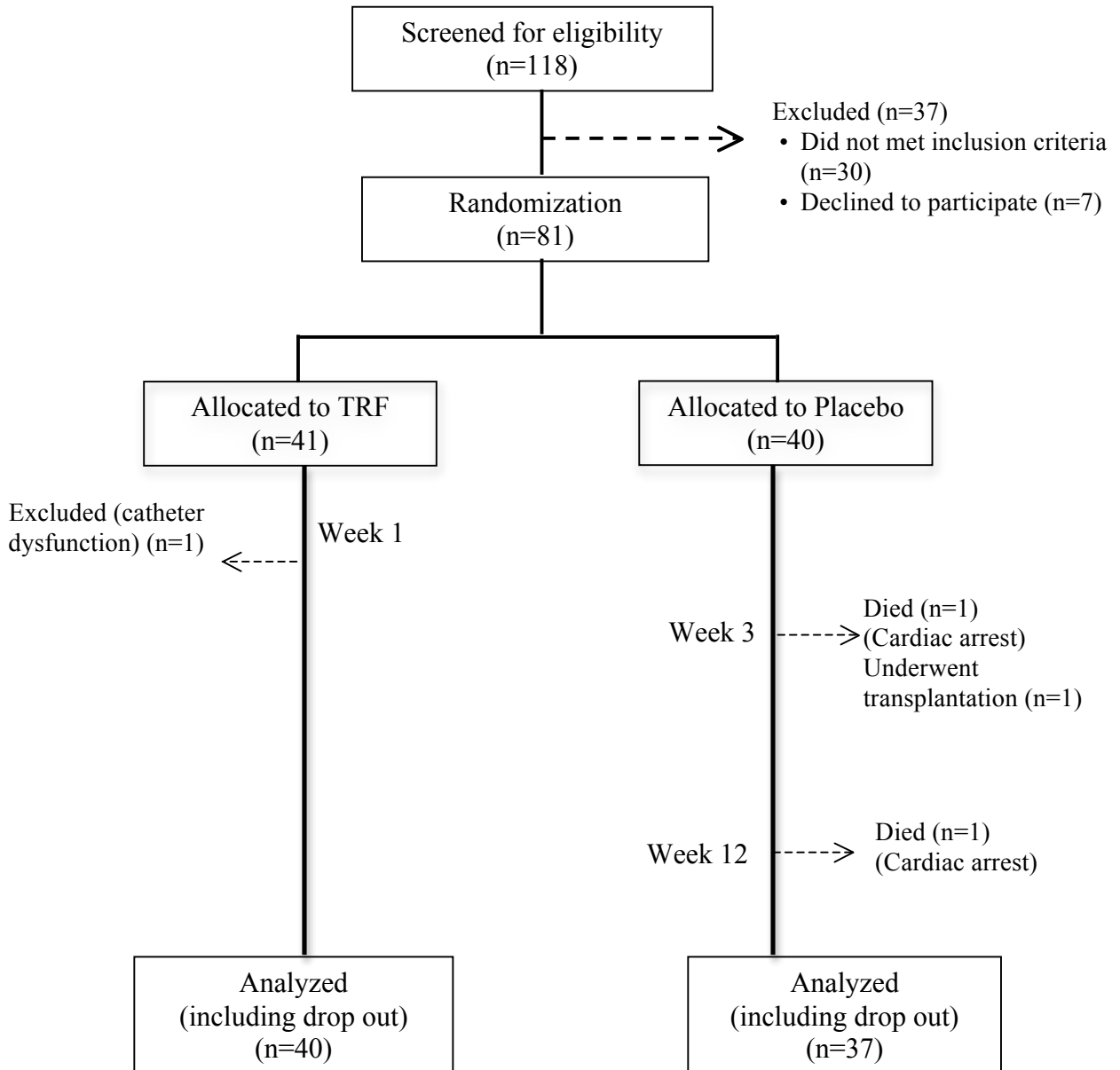
Note: All patients (n=118) were screened for eligibility. A patient was excluded if he/she was <18 years age, undergoing dialysis treatment <3 months, resident of nursing homes, undergoing 8 hours of dialysis treatment or receiving intradialytic parenteral/enteral feeding. Data were analyzed according intention-to-treat regardless of post-randomization withdrawal.

**FIGURE 5-2.** Dietary supplements intake among study's subjects.
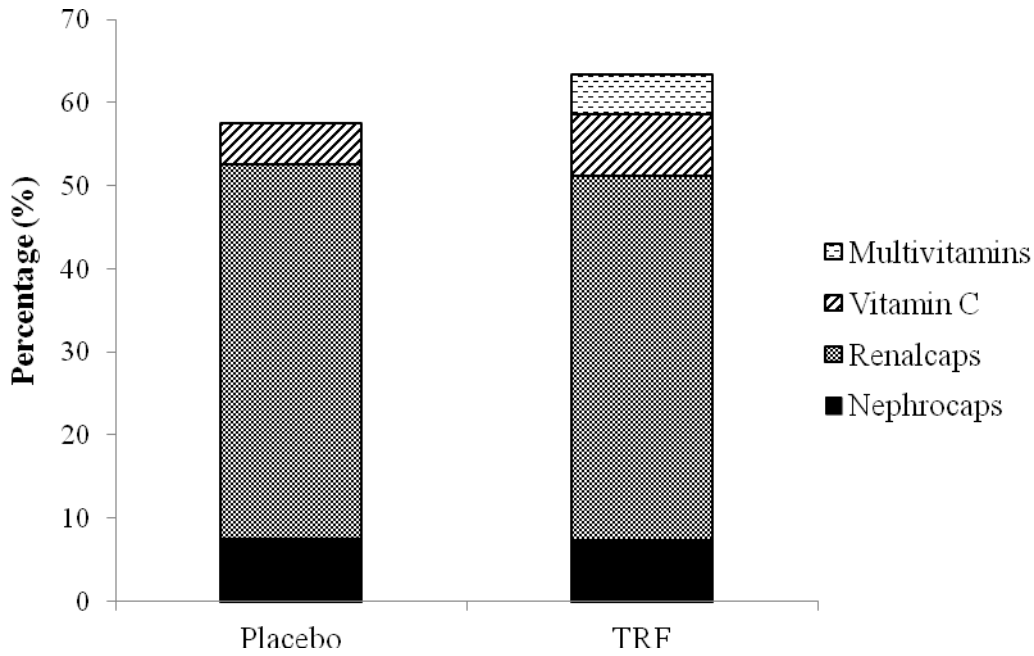
Note: This figure shows dietary supplements intake as reported by the study's subjects. Values are presented as percentage of patients taking particular supplements during the study period. Neprhocaps and Renalcaps (generic) contain the same amount of vitamins as follow: 100mg vitamin C, 1.5mg Thiamine, 1.7mg Riboflavin, 20mg Niacin, 10mg Vitamin B6, 1 mg Folate, 6μg vitamin B12, 150μg Biotin and 5mg pantothenic acid.

FIGURE 5-3. Percent of capsule 'consumption' in placebo (n=38) and TRF (n=40) group during the course of the study.
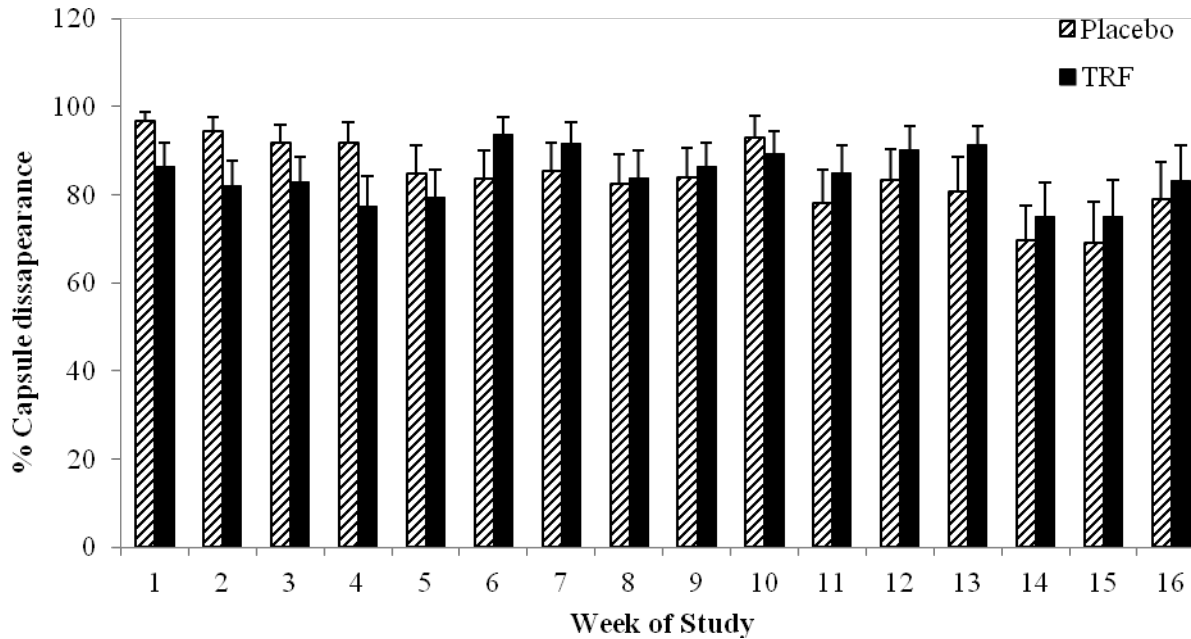

Note: This figure shows percent of capsule consumption (disappearance) for non-dialysis days, measured by pill counting method for 16 weeks of the study. Values are expressed as mean±SEM. No significant differences for each week between the two groups were noted as tested by independent t-test.

FIGURE 5-4. NFκB p65 value at baseline and week-12.
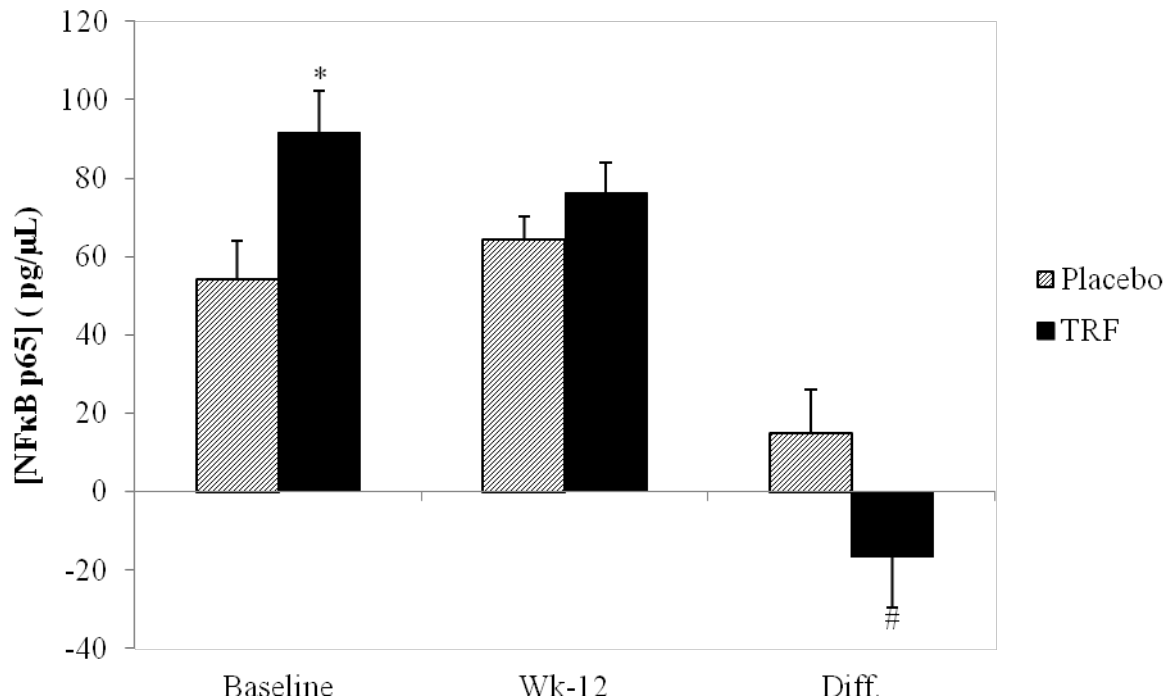
Abbreviations: NFκB, nuclear factor kappa B; Diff, mean difference in [NFκB] (wk-12 minus baseline).

Note: This figure shows NFκB p65 concentration measured in the nuclear extract using ELISA-based kit. Values are reported as mean±SEM. Independent t-test was used to test the mean differences between TRF and Placebo groups at each time point (baseline, wk-12, diff). Symbol * indicates significant difference (p<0.05) while # indicates marginal difference (p=0.08) when compared to the placebo group.

FIGURE 5-5. TNFα value at baseline and week-12.
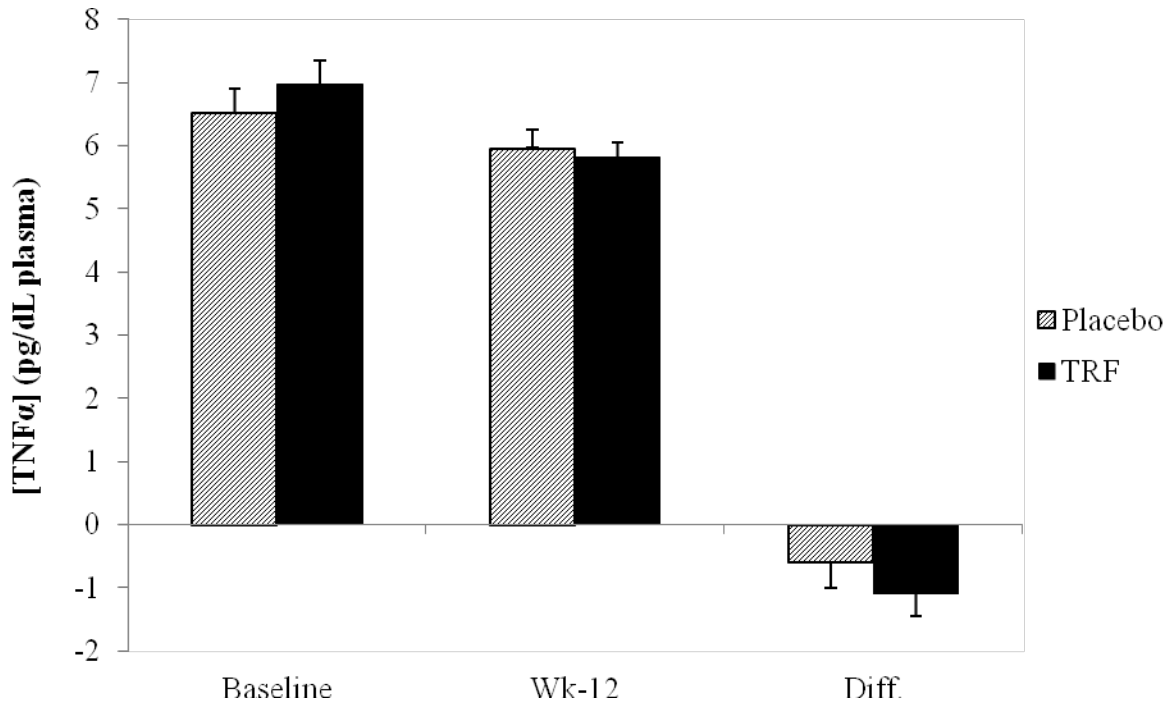
Abbreviations: TNFα, tumor necrosis factor alpha; Diff, difference.

Note: This figure shows TNFα concentration measured in the plasma using ELISA-based kit. Values are reported as mean±SEM. Independent t-test was used to test the mean differences between TRF and Placebo groups at each time point (baseline, wk-12, diff). No different was noted between placebo and TRF groups at all time points.

FIGURE 5-6. Mean changes in plasma TAG normalized to the baseline.
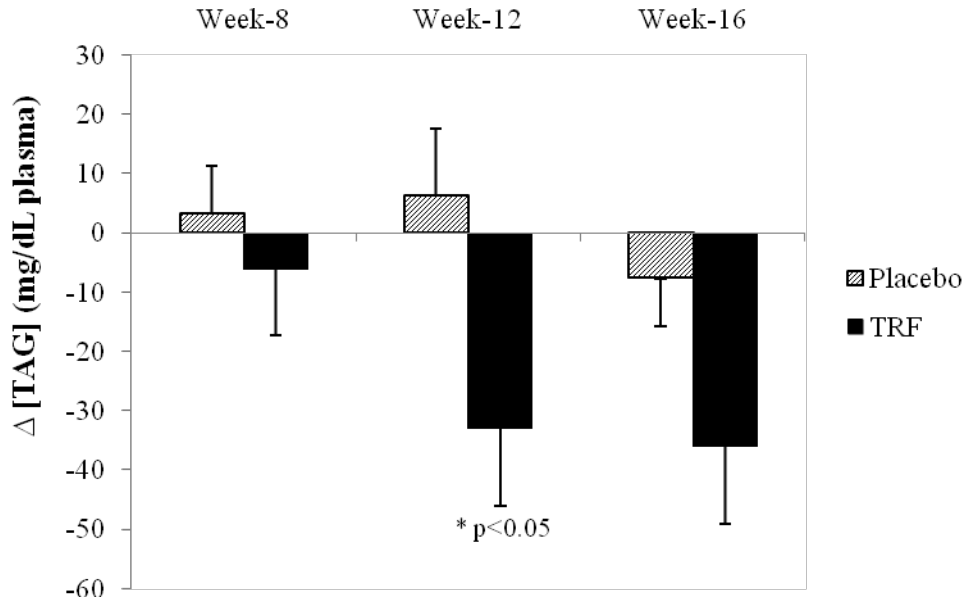
Abbreviations: TAG, triacylglycerols.

Note: Values are presented as mean±SEM. TAG data are calculated by different from baseline values. Symbol * indicates significant difference ($p < 0.05$) as compared to placebo at each time point based on independent t-test. At week-16 the there was a marginal different between TRF and placebo groups ($p = 0.072$).

FIGURE 5-7. Mean changes in plasma HDLC normalized to the baseline.
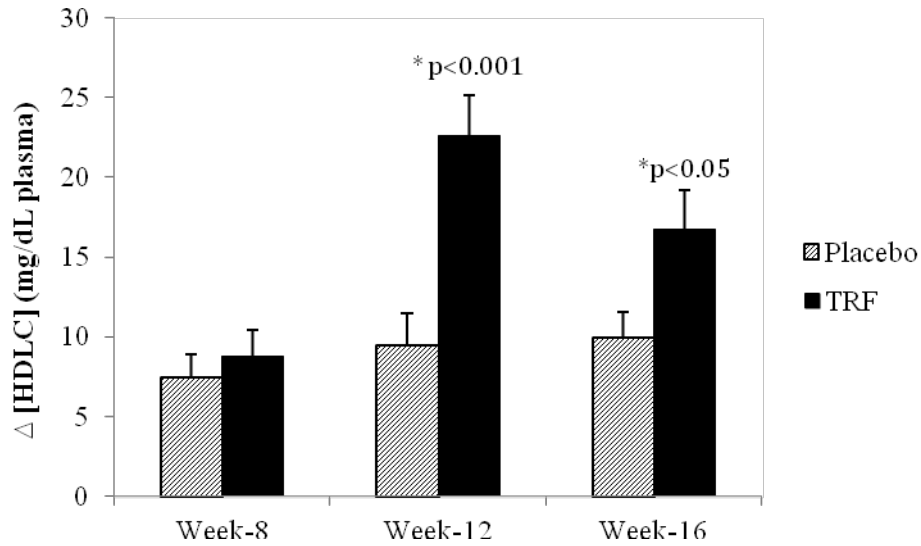
Abbreviation: HDLC, high-density lipoprotein cholesterol

Note: Values are presented as mean±SEM. HDLC data are calculated by different from baseline values. Symbol * indicates significant difference ($p < 0.05$) as compared to placebo at each time point based on independent t-test.

FIGURE 5-8. Change in TAG levels among statin and non-statin users in placebo and TRF groups.
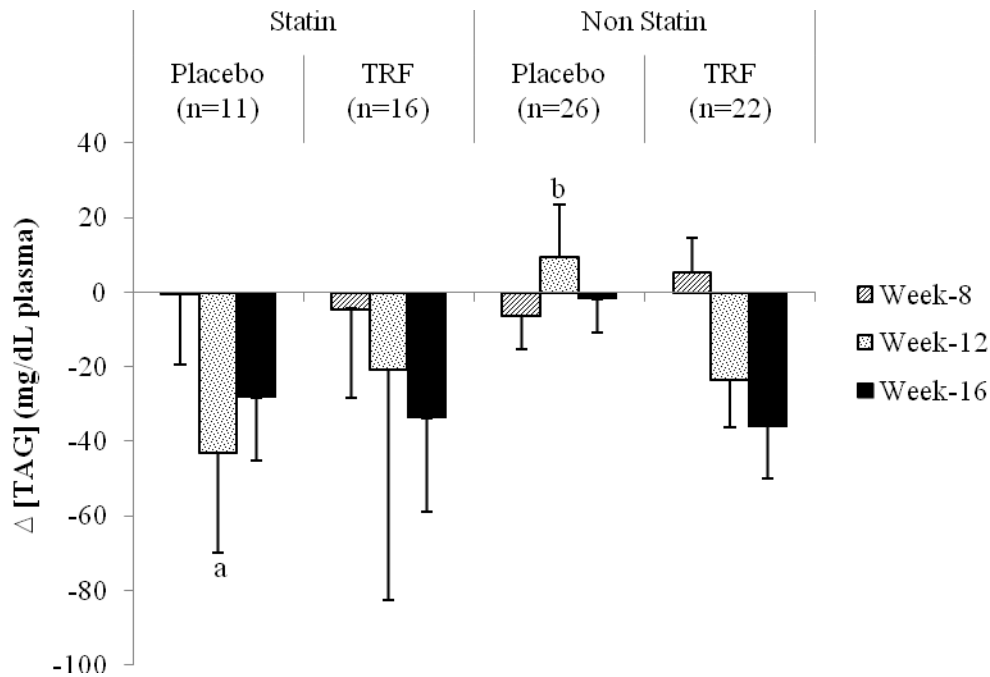
Note: Changes in TAG and HDLC were calculated by deducting baseline values. Data are reported as mean±SEM. Difference alphabet denote marginal difference (p<0.08), tested by independent t-test.

FIGURE 5-9. Comparison of ApoA1 (A) and CETP activity (B) during week-12 and week-16.
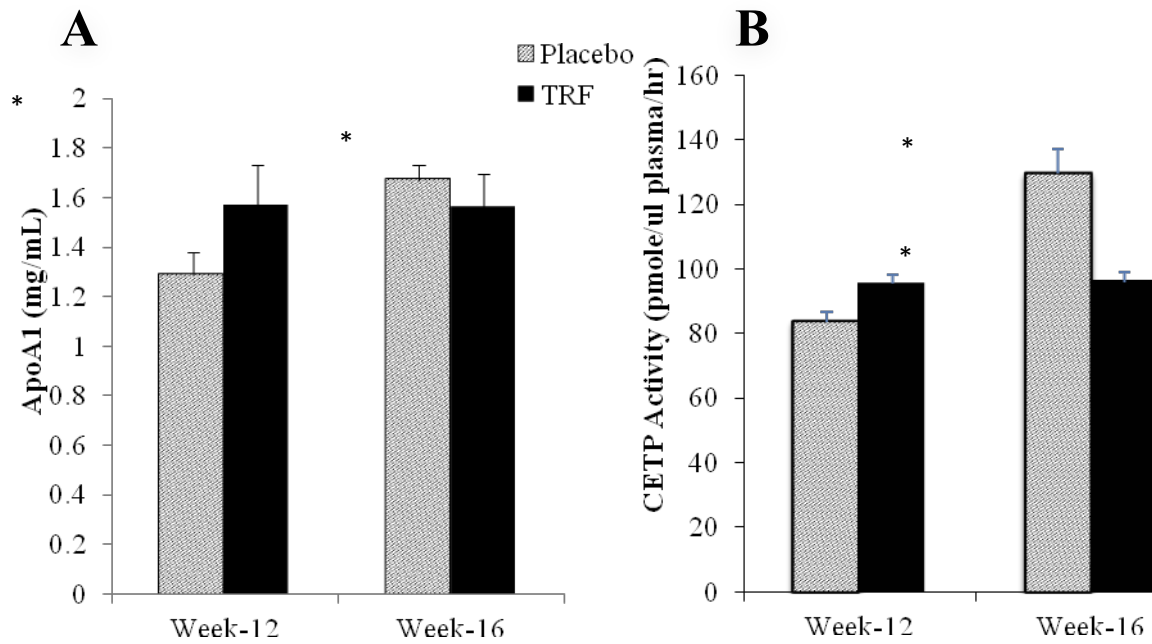
Abbreviations: ApoA1, apolipoprotein A1; CETP, cholesteryl ester transfer protein

Note: Data are presented as mean±SEM. Symbol * indicates significant difference ($p < 0.05$) as compared to placebo at each time point, tested by independent t-test. Figure A shows plasma ApoA1 concentration during week-12 and week-16 in both groups as measured by ELISA method. Figure B shows CETP (cholesteryl ester transfer protein) activity during week-12 and week-16in both groups as measured in the plasma using flourometric assay kit.

FIGURE 5-10. Pearson's correlation coefficient in lipid parameters during week-12 and week-16.
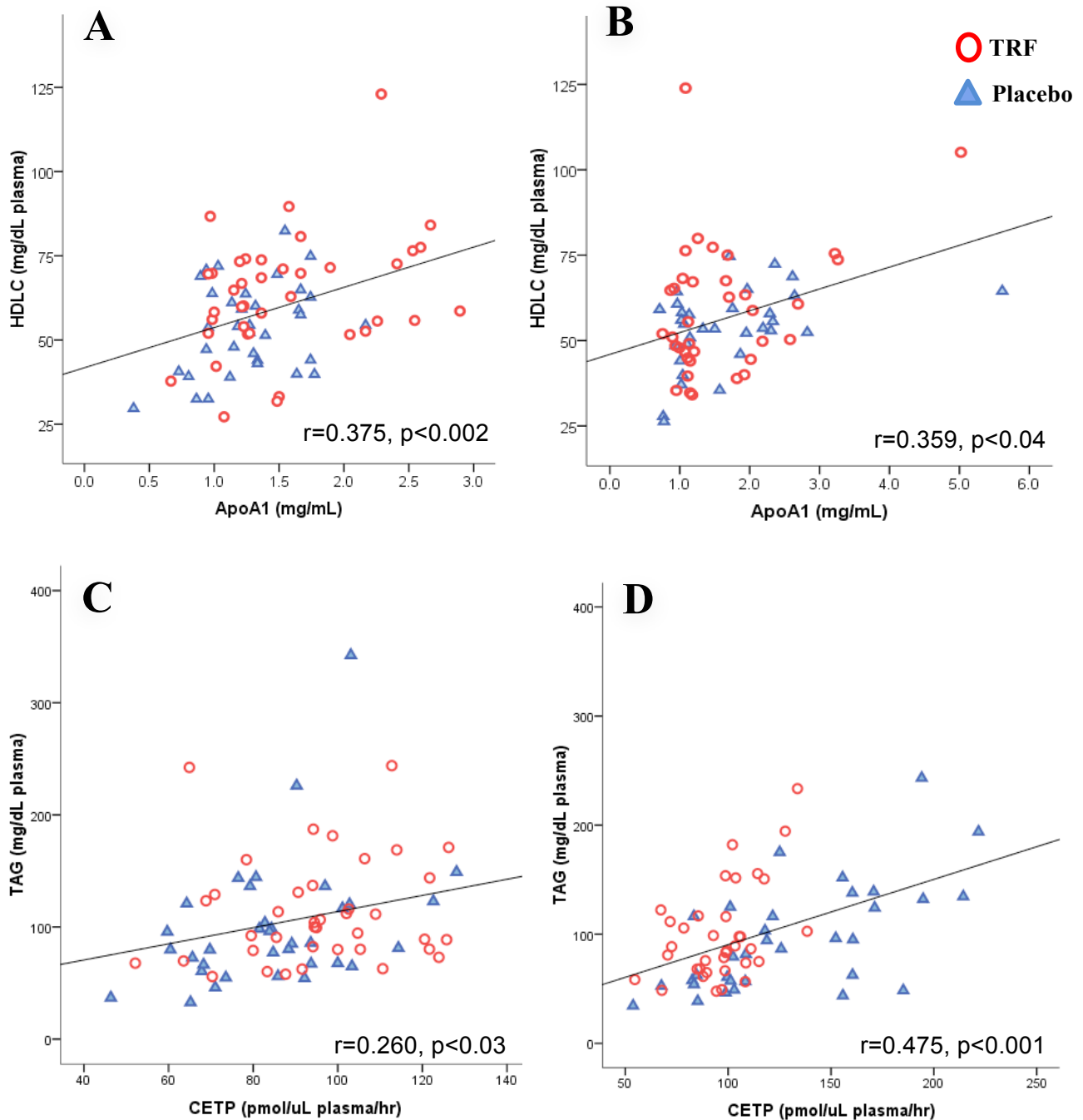
Abbreviations: HDLC, high-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; TAG, triacylglycerols; CETP, cholesteryl ester transfer protein.

Note: (A) (B) Correlation between ApoA1(mg/mL plasma) and HDLC (mg/dL plasma) at wk-12 and wk-16, respectively. (C) (D) Correlation between CETP activity (pmol/μL plasma/hr) and TAG (mg/dL plasma) at wk-12 and wk-16, respectively.

FIGURE 5-11. Cumulative days of missed treatment in placebo (n=38) and TRF (n=40) group
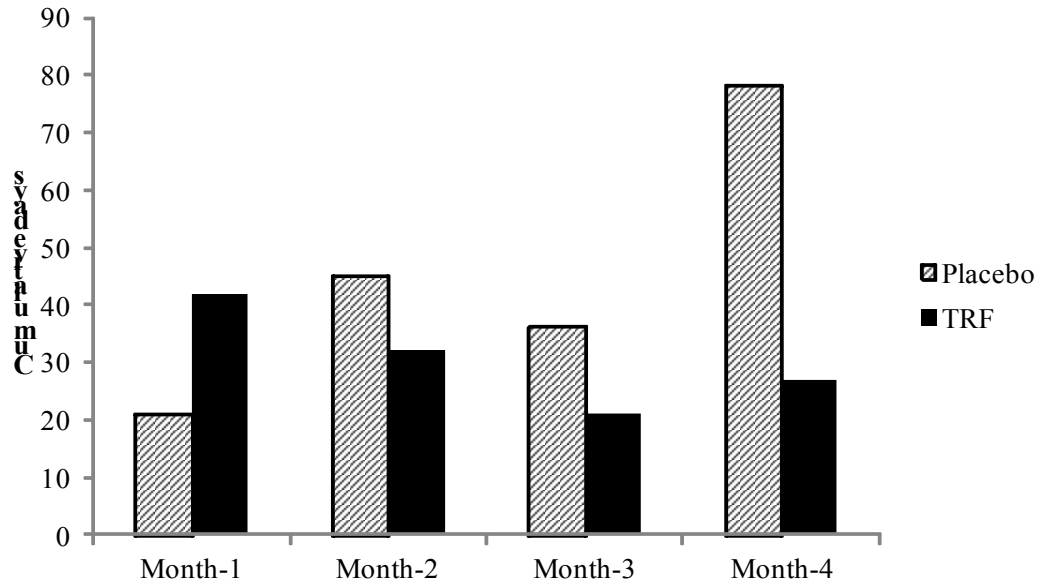
Note: This figure is for illustrative purpose only. Data are presented as absolute number of days of missed dialysis session during each month of study course. The total number of days missed was 122 *vs* 180 days for the TRF and placebo groups, respectively.

CHAPTER VI

SPECIFIC AIM 3:

CHANGES IN METABOLOMIC PROFILES FOLLOWING TRF

SUPPLEMENTATION

Introduction

ESRD patients on chronic HD have alarmingly higher risk for cardiovascular mortality when compared to the age-matched general population (i.e. 15-30 times higher risk) [3, 5]. In fact, cardiac death account for more than 50% of all-cause mortality in this population [209]. It is believed that malnutrition-inflammation complex syndrome, oxidative stress and dyslipidemia are implicated in the pathogenesis of cardiovascular diseases in this population [11, 12, 210]. Therefore, it is of interest to examine dietary impact of nutrients possessing antioxidant, anti-inflammatory or lipid altering properties to alleviate these problems in the HD population. One of the dietary compounds that have gained increasing scientific scrutiny is tocotrienols (TT). TT have been shown to confer cardio protection via its antioxidative, lipid-lowering, anti-inflammatory and anti-atherosclerotic properties in animal model as well as in human [34, 35, 37, 114].

However, the impact of such dietary intervention in a diverse clinical population is challenging to interpret partly due to intra- and inter-individual variability in response to the intervention, underlying comorbidities and the implication of compliance/non-compliance to the overall study's results. Moreover, the nature of traditional biomarkers such as lipid profiles (plasma levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol) may not adequately provide a complete picture of a disease progression because they are restricted to

a known pathophysiologic pathway or reflect only on a specific aspect of the disease process [152]. Fortunately, with the emerging of 'omics' technology especially metabolomics, an unbiased approach to study the breadth of metabolite disturbance following a disease state has become possible [39, 40]. The omics approach has allowed scientists to characterize a complex myriad of metabolic pathways involving lipids, sugars, organic acids, amino acids and other small molecule analytes that are the substrates or byproducts of metabolism, or have been ingested [152].

However, given the infancy age of the omics approach, there are relatively few literatures pertinent to HD patients. Metabolomics has been used to identify potential cardiovascular risk biomarkers in several groups [152, 237]. For example, Senn and colleagues studied a large cohort of patients undergoing coronary angiography and found that several metabolites such as trimethylamine-N-oxide (TMAO), choline and betaine had a dose-dependent relationship with regards to the presence of CVD and the severity of atherosclerotic heart diseases [152]. Interestingly, in CKD patients not on dialysis, NMR studies of plasma and urine indicates accumulation of aliphatic amines including TMAO [238]. In a recent study, Fujiwara and colleagues utilized metabolomics for pattern recognition analysis to differentiate plasma metabolic profile of pre- versus post- HD treatment. They found that the NMR spectral pattern of plasma between pre and post HD treatment was clearly discriminated. Upon identification and quantification of the metabolites, they found that significant fluctuations were seen in the levels of creatinine, TMAO, glucose, lactate and acetate when comparing pre- and post- HD plasma metabolites [239].

In Chapter V, we reported that TT administration in the form of TT-rich fraction (TRF) in a randomized, double-blinded, placebo-controlled, parallel trial for 16 weeks improved lipid

profiles in patients undergoing chronic HD as characterized by lower TAG and higher HDLC in the TRF group when compared to the placebo group. This was correlated with higher ApoA1, the main protein component of HDLC, while CETP activity, the plasma protein that facilitates the transport of cholesterol and TAG between lipoprotein was lower in TRF group. Although there were some reduction trends in inflammatory markers and oxidative status parameters, none of these achieved statistical significance. Interestingly, TRF group also had relatively less hospitalization days when compared to the placebo groups. We speculate that no significant changes were seen in inflammatory markers due to high intra and inter-individual variability of these markers due to a dynamic response of the immune system, in-concert with other studies [224, 226]. Hence, such study requires a relatively bigger sample size to increase the statistical power in order to detect the difference [240]. Therefore, the objective of this specific aim was to determine whether the TRF supplementation led to overall changes in patients' metabolomics profile and whether the observed changes in plasma lipids correlate with their metabolomics profile. In order to achieve this specific aim, ^{1}H nuclear magnetic spectroscopy (^{1}H NMR) was utilized to simultaneously analyze numerous metabolites detectable in patients' plasma sample, to provide an insight in metabolomics shift overtime and their differences between treatment and placebo groups. A powerful multivariate data analysis (MVDA) using statistical model such as principal component analysis (PCA) and partial least square (PLS) was used to analyze this data set.

Methods

Subjects and study design

This experiment is a subset of the previously reported study (Chapter VI and Daud et al 2013 [208]). The study was approved by the Human Investigation Committees of Wayne State

University and Great Lake Dialysis, LLC. Procedures for patient selection and randomization were as reported in Chapter VI. Patients were randomly allocated into treatment group (TRF) or placebo, and received the respective supplementation for 12 weeks. The study was extended for another 4 weeks (for a total of 16 weeks) to confirm some substantial changes observed in week-12. All of our patients were undergoing dialysis with the same high-flux dialyzer membrane (Fresenius Optiflux Dialyzer, Fresenius Medical Care, Waltham, MA, USA). Both groups had a comparable dialysis adequacy (Kt/V) (placebo 1.45±0.20; TRF 1.48±0.26, p=0.516) as estimated by single-pool model.

Blood sample collection and preparation for NMR analysis

Approximately 5mL of fasting (for ~10 hrs) midweek predialysis blood samples were collected from existing access sites of each study participant into blood collection tubes containing lithium heparin (Becton Dickson, Franklin Lakes, NJ, USA) at baseline and week-12. Plasma was isolated by centrifugation at 2800rpm for 20 minutes at $4^{o}C$, divided into aliquots and immediately stored at $-80^{o}C$ until further analysis.

For NMR analysis, plasma samples were thawed and equilibrated at room temperature. Upon thawing, plasma samples were centrifuged at 10,000 rpm for 2 minutes to remove solid debris. An aliquot of 400μL of plasma supernatant were transferred into an assembled Amicon Ultra 0.5mL 3kDa filter device (Sigma-Aldrich, St. Louis, MO, USA) that had been pre-washed three times with deionized water (450μL deionized water, spun for 13,800 rpm for 5 minutes each wash). The filter devices containing plasma were centrifuged for 30 minutes, 13,800 rpm at room temperature. The 'filtrates' were subsequently transferred into new microtubes and were diluted with deuterium oxide (D_2O) (Cambridge Isotope Inc., Andover, MA, USA) in 1:1 ratio

(300μL filtrate + 300μL D2O). NMR cocktail containing 5mmol/L 3-(trimehylsilyl)-1 propanesulfonic acid (TMS) (Sigma-Aldrich, St Louis, MO, USA) and 10mmol/L Imidazole (ACROS Organics, Thermo Fisher Scientific, NJ, USA) was added to the diluted plasma samples in 9:1 ratio. The prepared samples were subsequently transferred to 5mm NMR tubes (8 inches long) (Sigma-Aldrich, St. Louis, MO, USA). The summary of sample preparation for NMR analysis is illustrated in **Figure 6-1**.

Proton ^{1}H NMR acquisition

One dimensional ^{1}H NMR spectra were acquired using a 600MHz Agilent spectrometer, performed at a temperature of 27°C. The free induction decays (FIDs) were collected into 32K datapoint, for a total of 64 scans, with a spectrum width of 10 ppm, and an acquisition time of 4 seconds. The water signal was suppressed by a presaturation and set the flip angle to 90°.

Spectral processing

FIDs were processed with ACD software version 12.0 (Advance Chemistry Development, Inc., Toronto, ON, Canada) by stacking together as a group, followed by Fourier transformation, auto-phasing and auto-baseline correction. The whole spectra were divided into 1000 equal bins using intelligent binning option. The spectra were digitized into a table of common integrals containing a non-negative value and exported as a text file for multivariate data analysis. **Figure 6-2** summarized the spectral processing procedure using ACD software.

Multivariate data analysis

Multivariate data analysis was performed using SIMCA-P 12.0+ (Umetrics, Umea, Sweden). Briefly, the data text file was imported into the software and transposed. Given that, each row

represents a case (i.e. a patient) while each column represents a variable (i.e. 1000 binned spectra) (**Figure 6-3**). Data was statistically analyzed using three types of mathematical algorithm, namely 1) principle component analysis (PCA), 2) partial least square (PLS) and 3) PLS-discriminant analysis (PLS-DA). PCA is an unsupervised statistical procedure that is designed to display the systematic variation in the data matrix X – this is achieved by showing related observations and deviations/similarities in the data set via a score plot and a loading plot. PCA is often used for exploratory analysis of the data set. The score plot provides visual information regarding the similarities/differences in the data while the corresponding loading plot provides information regarding the variables (the part of spectrum) that is responsible for the difference/similarities observed in the score plot [151]. PLS and PLS-DA is a supervised method of analyses. PLS is basically an extension of PCA in which Y variable(s) is added to connect with the information provided by X variables. In addition, PLS-DA identify the model that separates the classes of observation based on X variables and hypothetical Y variables. All x variables were Pareto's scaled before multivariate data analysis.

Metabolites identification and quantification

Loading plot of PCA provides a visual indication on the part of the spectra and corresponding metabolites that are responsible for the differences in metabolic profile between the two groups. These metabolites were identified and quantified using Chenomx NMR 7.1 suite (Edmonton, Alberta, Canada). Briefly, FID files were first processed in the Chenomx Processor to adjust for pH, assign chemical shift indicator and remove line shape distortion by autophasing, autospline baseline correction and reference deconvolution. The processed spectra were then exported into Chenomx Profiler for identification and quantification of the metabolites. There were 313 known metabolites in the Chenomx Profiler (using 600Mhz magnet database) that is specific for

different magnetic strength and pH ranges. Each metabolite and their corresponding peak clusters were matched to the existing database and the area under the peak dictates the concentration of that particular metabolites (**Figure 6.4**). Data on these quantified metabolites were exported into SPSS for statistical analysis.

Statistical analysis

Apart from multivariate data analyses as explained above, statistical analyses for the identified metabolites were carried out using SPSS v.16 (IBM, Chicago, IL, USA). Independence t-test was applied to test the difference in the metabolites concentration between TRF and placebo groups. P values of <0.05 is regarded as statistically significant.

Results

Metabolomics profile at baseline

PCA was conducted at baseline to investigate whether the metabolomics profiles between TRF and placebo groups were different. The PCA-X score plot at baseline indicates that metabolomics profiles between the two groups were not different as evident by the scattered and random scores from both groups in the eclipse. There was no particular pattern observed at this point (**Figure 6-5**).

Metabolomics profile at week-12

The same analysis (PCA) was performed at week-12 to investigate the difference in metabolomics profiles at week-12. PCA-X plot indicates that there was some pattern of scores between the two groups; however, no clear separation was observed (**Figure 6-6**). Further analysis using PLS-DA indicates that there was a clear separation between TRF and placebo

groups in the PLS-DA score plot by the principal component 1 (**Figure 6-7**). Hence, this is suggesting that TRF supplementation induced specific metabolomics pattern change resulting in different profiles.

Correlation of metabolomics profiles with other parameters

Two-dimensional PLS plots were also performed to investigate predictive effects of plasma metabolome in relation to known biomarkers. **Figure 6-9** shows correlation between metabolomics profile (x-axis) and inflammatory markers (CRP, IL-6) (y-axis) of the two groups at week-12. There was a weak correlation (R^2=0.38) between metabolomics profile and inflammatory markers. In other words, the PLS regression between the plasma metabolome from NMR and plasma inflammatory markers indicate that 38% of the variations in the inflammatory markers can be predicted by the treatment conditions (placebo *vs* TRF).

Similarly, the PLS analysis between plasma metabolomics profiles and plasma oxidative status (TAP and MDA) revealed a weak correlation (R^2=0.26) (**Figure 6-10**). This may indicate that plasma TAP and MDA have less predictive power towards the changes in metabolomics profile.

Meanwhile, plasma HDLC (**Figure 6-11**) and lipid profiles (TC + TAG + HDLC) (**Figure 6-12**) were also moderately correlated with plasma metabolomics profile (R^2=0.49 and R^2=0.60, respectively) indicating that the observed change in plasma HDLC and lipid profiles were reflected in the change in concentration of metabolites present in the plasma. Based on this result, 60% of the variations in the lipid profiles can be predicted by the treatment group allocation. This is also in concert with the fact that most changes in lipid profile were seen at week-12 of the intervention.

Metabolites identification and quantification

PCA loading plot (**Figure 6-8**) provides a guide on the part of the spectra that is responsible for the differences observed between TRF and placebo groups at week-12. By using Chenomx NMR Suite software, a total of 33 metabolites were identified and quantified. These metabolites were significantly different ($p<0.05$) or approached significance ($0.05<p<0.10$). However as the metabolites were analyzed from 4 subjects per treatment – the fact that some metabolites were at the lower limit of detection in one or two subjects per treatment, invariably skewed group means in certain cases. Regardless, some of these metabolites included various compound classes such as amino acids (e.g. histidine, methionine), carbohydrates (e.g. galactose, lactose), organic acids (e.g. lactate) and phospholipids related compound. Some of the metabolites that have been previously shown to be implicated in CKD included 2-Hydroxyglutarate, 3-Hydroxy-3-methylglutarte, Betaine, Cadaverine, Glycerate, Mandelate, Proline and 2-Hydroxy-3-methylvalerate as well as Citrate, N-acetylaspartate, N-acetylcysteine and Ribose. However until analyses from more subjects is completed no definitive statement or conclusion can be made.

Discussion

In this experiment, we showed that plasma metabolomic profiles of HD patients between TRF and placebo group did not show any different at baseline. After 12 weeks of supplementation, initial analysis using unsupervised PCA of the plasma metabolic profiles did not indicate obvious treatment related effects, with only some pattern of separation at principle component 1. However, by using PLS-DA modeling, TRF group's plasma metabolomic profile was clearly discriminated from the placebo group. The fact that plasma metabolomic profiles were different between the two groups after 12 weeks of intervention may indicate 1) real effects of TRF that

induced metabolic changes reflected in the plasma metabolic profiles, or 2) potential confounders such as medications, dietary intake and life style, that could influence metabolic changes. To complicate things, there are also lines of evidence reporting the impact of gut bacteria on the metabolome [148].

It is unlikely that dietary intake/pattern of a stable chronic dialysis patient had changed partly because of diet monotony due to dietary restrictions, which have been consistently reported in the literature [32, 241, 242]. However, it is possible that diversity in medication profiles of HD patients may contribute to variations in metabolic profiles, resulting in artifact and misinterpretation. Given the significant co-morbidity experience by HD patients, many of our patients were on a handful of medication such as anti-hypertensive drugs (e.g. atenolol, amlodipine, losaratan), blood-thinning medication (e.g. aspirin), diuretics (e.g. furosemide), lipid-lowering drug (e.g. statins), and phosphate binders (e.g. calcium acetate). It is almost impossible to control on this aspect given the 'intention to treat' nature of the clinical study. In order to overcome such variations, one may require a relatively larger sample size as compared to animal, or cell metabolomics studies [150], but this may not be feasible. Therefore, the preferred strategy to overcome this variability is to perform a small-scale untargeted approach as outlined by Mamas and colleagues:

> "*In metabolomic studies of the general population, the preferred strategy is to perform two independent small-scale holistic studies (defined as discovery and validation) where the number of samples is typically 10–100s and where many potential confounders (e.g. age, BMI, gender) are controlled so that the only 'random' variable between classes is the disease or drug treatment. These studies define potential biomarkers or disease pathophysiology*"
> Mamas et al. (2011) [150].

Given limited funding and time constraints, we streamlined this experiment in order to evaluate the actual effects of TRF by selected patients with similar medication and demographic

profiles. As discussed earlier, we found that the metabolomic profiles of TRF and placebo groups were clearly discriminated at week-12 but not at baseline; suggesting TRF induced metabolic pattern change. Furthermore, based on two-dimensional PLS analysis, metabolomic profile of the patients at week-12 was significantly correlated with lipid profiles in-concert with the changed in lipid profile measurements. Interestingly, PLS tests also indicate that plasma metabolomics profiles were correlated with serum albumin and inflammatory markers. This is an intriguing observation given the fact that we did not see any difference in serum albumin and inflammatory markers [208] which may suggest the need for a bigger sample size due to high variability in these traditional biomarkers. However, the correlation between metabolomics profiles and inflammatory biomarkers may need to be viewed in relation to anti-inflammatory properties of aspirin due to the fact that all subjects in this subset analysis were prescribed aspirin (acetylsalicylic acid). Interestingly, removal of aspirin peak clusters (2.32, 7.13, 7.145, 7.35, 7.37, 7.50, 7.51, 7.52, 7.67, 7.68ppm) in the spectra database from the SIMCA software prior to MVDA analysis did show the same pattern of discrimination, suggesting the minimal effects of aspirin as anti-inflammatory agent.

Based on the preliminary metabolites identification data, several metabolites that were identified appear to be involved in CKD. Betaine is a metabolite of choline and recent evidences indicate that increased levels of choline, betaine and TMAO are associated with increased risk for cardiovascular diseases [152, 243]. 3-hydroxy-3-methylglutarate (HMG), is a metabolite that accumulates in the urine of patients affected by 3-Hydroxy-3-methylglutaric aciduria. Recent evidence indicates that accumulation of HMG in the plasma is associated with lipid and protein oxidative damage as well as reduce enzymatic antioxidant defense in rat cerebral cortex [244]. It

is postulated that the accumulation of HMG in ESRD patients may be due to residual uremia because dialysis does not effectively remove uremic toxins.

Cadaverin and some other polyamines have been reported to increase in the late stage of CKD and in HD patients [245]. Accumulation of cadaverin is associated with suppression of erythropoiesis [246] leading to hyporensponsive of anemia treatment by Epogen (human erythropoietin produced via recombinant DNA technology), resulting in higher dosage requirement [245, 247]. This is exemplify by the fact that patients on peritoneal dialysis treatment are generally less anemic than those on HD [248, 249] due to a more efficient middle molecules removal [245, 248] and frequent dialysis (four times daily *vs* three times a week). It is interesting that recent publication indicate that supplementation with vitamin E improved Epogen responsiveness in HD patients [250], suggesting a potential target of vitamin E on uremic toxins that contribute to erythropoiesis suppression. Cruz et al reported that the use of vitamin E coated membranes in 172 maintenance HD patients leads to reduction of Epogen dosage and improved hemoglobin levels [251].

Because kidney plays an important role in the metabolism of protein and amino acids, kidney failure results in alteration of plasma amino acids (AA). It has been found that plasma concentration of citrulline, cysteine and taurine are increased in the early stage of CKD [252]. On the other hand, most plasma AA in the late stage of CKD and in ESRD undergoing HD treatment showe a decreased except for proline, asparagine, citrulline, asymmetric dimethylarginine and hydroxykynuerenine that show an increase in their levels [253]. This increased is hypothesized to be due to uremic retention and/or systemic over production. It has also been reported that the changes in plasma AA are associated with disease severity as indicated by the significant correlation in eGFR in a multivariate model [253].

Some other metabolites of interest in the initial anlayses include N-acetylcysteine and N-acetylaspartate. It is interesting that, levels of N-acetylaspartate and N-acetylaspartylglutamate (based on magnetic resonance spectroscopy of the brain) have been found to be reduced in dialysis patients, and these are associated with attention deficit, as well as disturbances in memory and learning activity [254]. This phenomenon has been postulated to be related to the accumulation of neurotoxic metabolites, hormonal disturbance and an imbalance in excitatory and inhibitory neurotransmitters following kidney failure [254, 255]. On the other hand, N-acetylcysteine confers antioxidant effect due to the presence of thiol groups in its molecular structure. N-acetylcysteine has been used as adjunct treatment during chemotherapy for the cancer treatment [256] and in the management of anemia and oxidative stress in HD patients [257]. Thus a complete analysis from the current sample set will yield important and exciting clues on the metabolic effects of TRF supplementation

Literature pertaining to metabolomics application in HD/ESRD patient is very scarce, therefore no *direct* comparisons are available. Only a few studies have explored the utilization of this technology especially for diagnostic purposes. For example, Sato et al [258] explored metabolomics analysis of plasma sample of pre- and post- HD treatment, and subsequently identified potential novel biomarkers that can be used as alternatives to urea and creatinine to determine dialysis dose adequacy. Fujiwara et al [239] utilized metabolomics approach for pattern recognition analysis and found that NMR spectra between pre and post HD treatment was clearly discriminated due to fluctuation in the levels of creatinine, TMAO, glucose, lactate and acetate. Interestingly, both of the studies did not report their patients' medication profiles.

Future directions

Based on the multivariate data analysis of the plasma metabolomics profile to date, it is now understood that supplementation with TRF triggers some metabolic changes which appear to be reflected in the plasma metabolites. Preliminary metabolite identification and quantification revealed several metabolites that responsible for the differences between TRF and placebo groups. These metabolites however need to be firstly confirmed and secondly, validated with biochemical assays for confirmation. Upon validating these metabolites, they should be compared to known biochemical pathways to examine their relevance in the disease process as well as in diagnostic and treatment aspects in relation to TRF supplementation.

Table and Figures

TABLE 6-1. Characteristics of the samples for metabolomic analysis.

	Placebo (n=9)	TRF (n=8)	P value
Demographics			
Age (yrs)	54	59	N.S.
African American (n, %)	9 (100)	8 (100)	N.S.
Gender (males, %)	5 (55.6)	5 (62.5)	N.S.
Clinical			
Diabetes Mellitus (n, %)	6 (66.7)	5 (62.5)	N.S.
Time on dialysis (months)	32	35	N.S.
BMI (kg/m^2)	29.3	27.9	N.S.
Kt/v	1.48	1.45	N.S.
Statins (n, %)	0 (0)	0 (0)	N.S.
Aspirin (n, %)	9 (100)	8 (100)	N.S.
Anti-hypertensive, one or more type (n,%)	9 (100)	8 (100)	N.S.

Note: Data are reported as mean for continuous variables and number (percentage) for categorical variables. P values are comparison between groups obtained from independent t-test or Pearson χ2 test wherever appropriate.

**FIGURE 6-1.** Sample preparation for NMR analysis
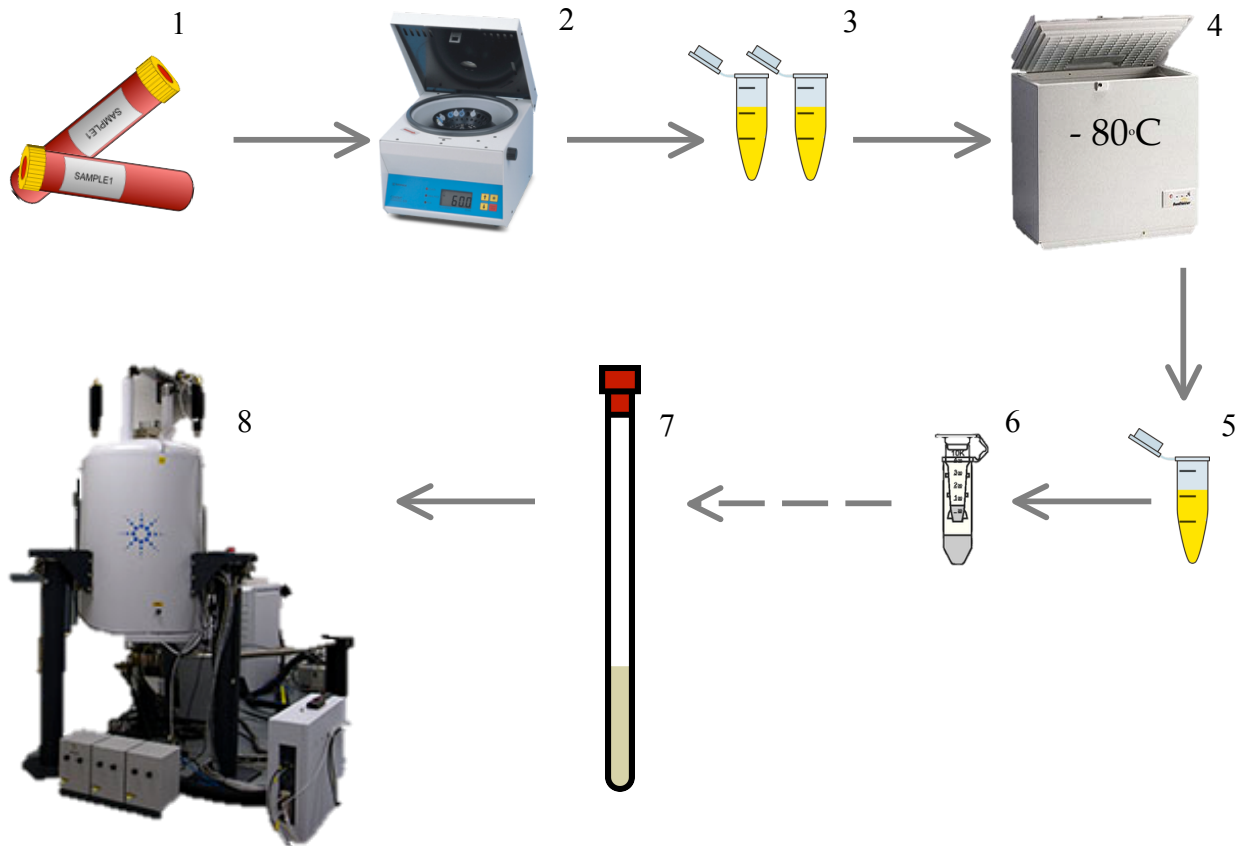
Note: This figure illustrates steps during sample preparation for NMR analysis: 1) Blood sample were collected into lithium heparin tubes, 2) Plasma was obtained by centrifugation, 3) Plasma was transferred into a clean micro tube for storage, 4) Sample was stored at -80°C until use, 5) Frozen plasma was thawed at room temperature, 6) High molecular weight compound was removed via 3kDa ultracentrifugation device, 7) 'Filtrate' was used for NMR analysis. During this step, sample was spiked with the internal standard (TMS) and pH indicator was added. 8) A total of 600µL sample (diluted 1:1 in deuterium oxide) was used for NMR FID acquisition.

FIGURE 6-2. Illustration for processing of NMR spectra using ACD software v. 12.0

All FID files acquired from 600 MhZ ^{1}H NMR were imported into ACD software. The x-axis represents the time domain.

The FID files were Fourier transformed followed by auto-phasing and auto adjustment for baseline. Reference peak (TMS) were also set at 0 ppm. The x-axis represents chemical shift (ppm).

The spectra were divided into 1000 equal bins. Each bin represents one variable.

The values from each bin are digitized into a non-negative numbers as a table of common integrals (TOCI). TOCI is then exported to SIMCA P+ software as a text file.

FIGURE 6-3. Multivariate data analysis processes using SIMCA-P 12.0+ software

Note: This figure shows a screen capture of multivariate data analysis (MVDA) processes from SIMCA-P 12.0+ software (Umetrics, Umea, Sweden). Upon importing table of common integral from ACD software, the data was transposed so that each column represents a variable while each row represents an observation (subject). The primary ID column (dark yellow) represents the particular subjects while the secondary ID dictates their group allocation. The data was further edited by removing corresponding variables that did not contain any peak on the spectrum (i.e. <0.5ppm and >8.0ppm), prior to running MVDA.

FIGURE 6-4. Metabolites identification and quantification using Chenomx software.
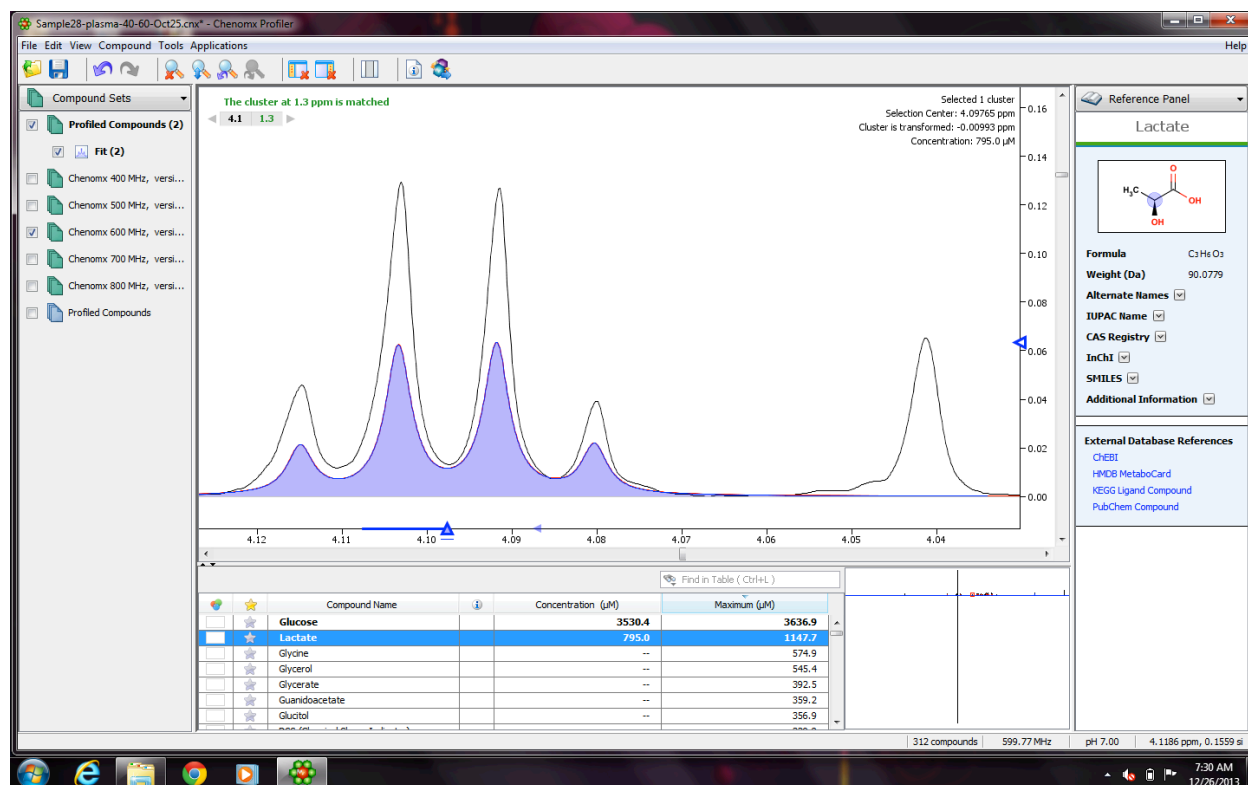
Note: This figure shows one of the processes of metabolites identification and quantification using Chenomx NMR Suite 7.7. The processed NMR file (following phasing, baseline correction and reference deconvolution) was exported into Chenomx Profiler. Each peak cluster in the NMR spectrum was manually matched with the database. In this example, lactate comprised of two peak clusters (i.e. at 4.1 and 1.3 ppm). Each peak cluster needs to be matched with the pattern of peak cluster in the database. The software will calculate the concentration of the particular metabolite based on the matched peak clusters area.

FIGURE 6-5. PCA score plot for NMR spectra obtained from plasma samples of HD patients at baseline.
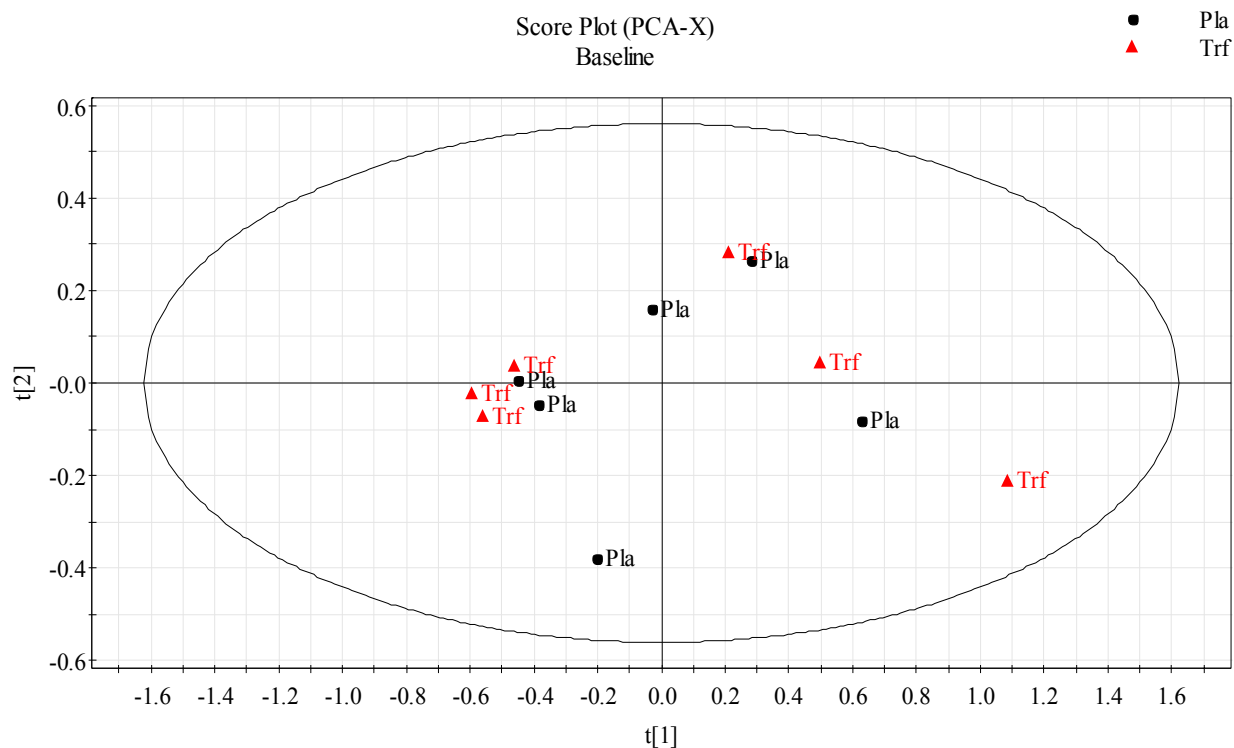
Abbreviations: PCA, principal component analysis; PLA, placebo; TRF, tocotrienol-rich fraction.

Note: This figure depicts PCA-X score plot indicating metabolomics profile between the two groups at baseline. The eclipse represents the 95th percentile of confidence interval, while any score outside of the eclipse is consider as an outlier. One outlier score was excluded from the analysis. Water peaks region (4.5 – 5.0 ppm) were removed from the data prior to analysis. The score for TRF and placebo groups were scattered indicating that the metabolomics profiles between the two groups were similar.

FIGURE 6-6. PCA score plot for NMR spectra obtained from plasma samples of HD patients at week-12.
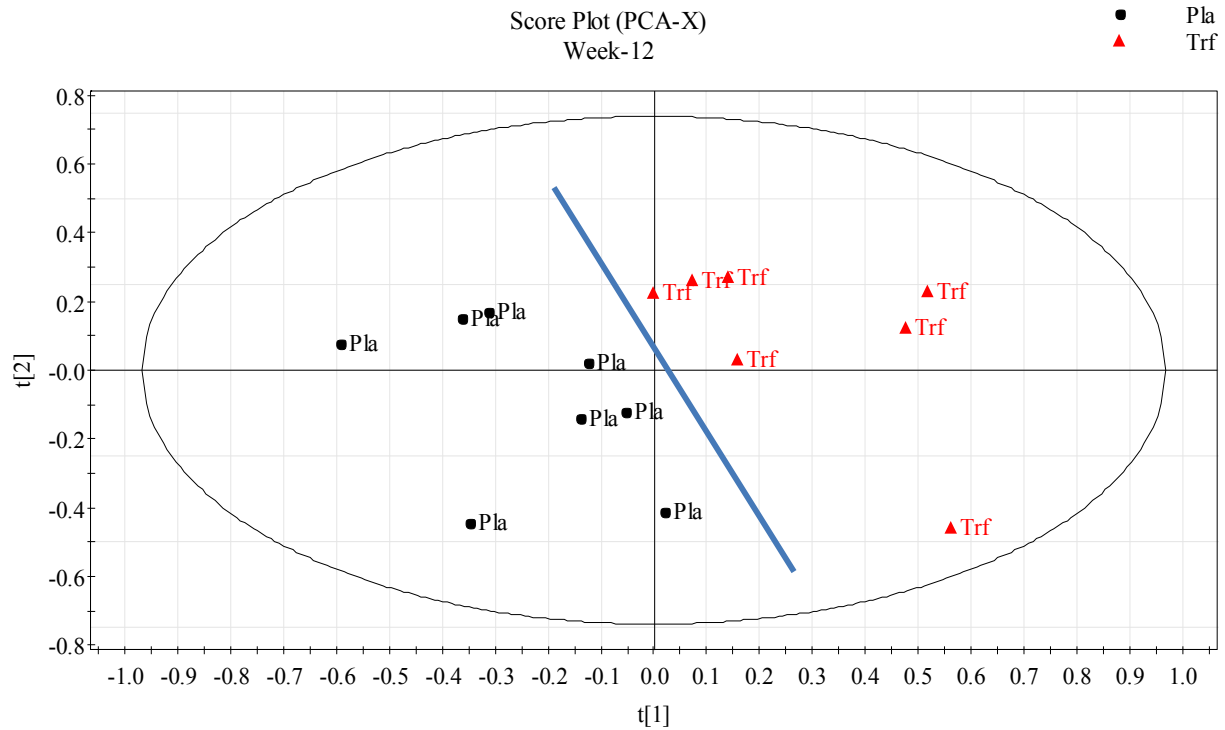
Abbreviations: PCA, principal component analysis; PLA, placebo; TRF, tocotrienol-rich fraction.

Note: This figure depicts PCA-X score plot indicating metabolomics profile between the two groups at week-12. The eclipse represents the 95th percentile of confidence interval, while any score outside of the eclipse is considered as an outlier. One outlier score was excluded from the analysis. Water peaks region (4.5 – 5.0 ppm) were removed from the data prior to analysis. The score plot shows there was separation trend indicating that metabolomics profiles of the two groups were discriminated.

FIGURE 6-7. PLS-DA score plot for NMR spectra obtained from plasma samples of HD patients at week-12.
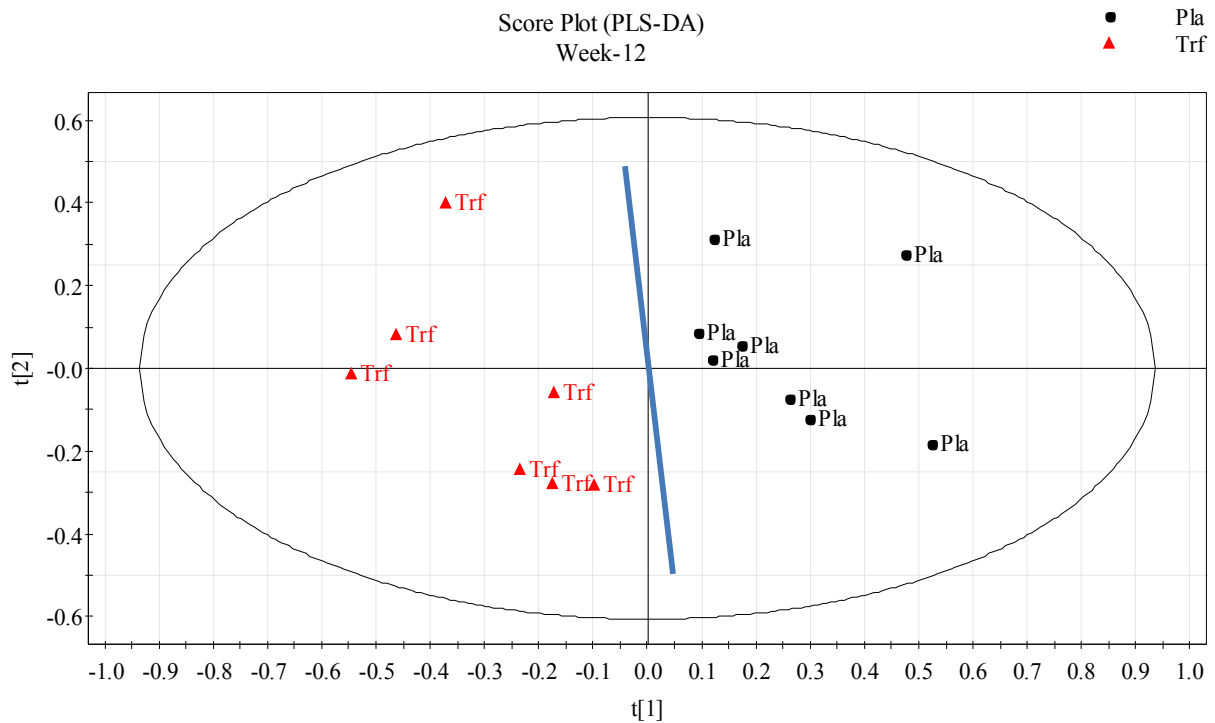
Abbreviations: PLS-DA, partial least square – discriminant analysis; PLA, placebo; TRF, tocotrienol-rich fraction.

Note: This figure shows PLS-DA score plot depicting metabolomics profile of the two groups at week-12. The eclipse represents the 95th percentile of confidence interval, while any score outside of the eclipse is considered as an outlier. One outlier observation was excluded from the analysis. Water peaks region (4.5 – 5.0 ppm) were removed from the data prior to analysis. Metabolomics profile of the two groups was clearly discriminated as indicated by a clear separation trend.

FIGURE 6-8. PCA-X loading plot for NMR spectra obtained from plasma samples of HD patients at week-12.

Loading Plot (PCA-X)
Week-12
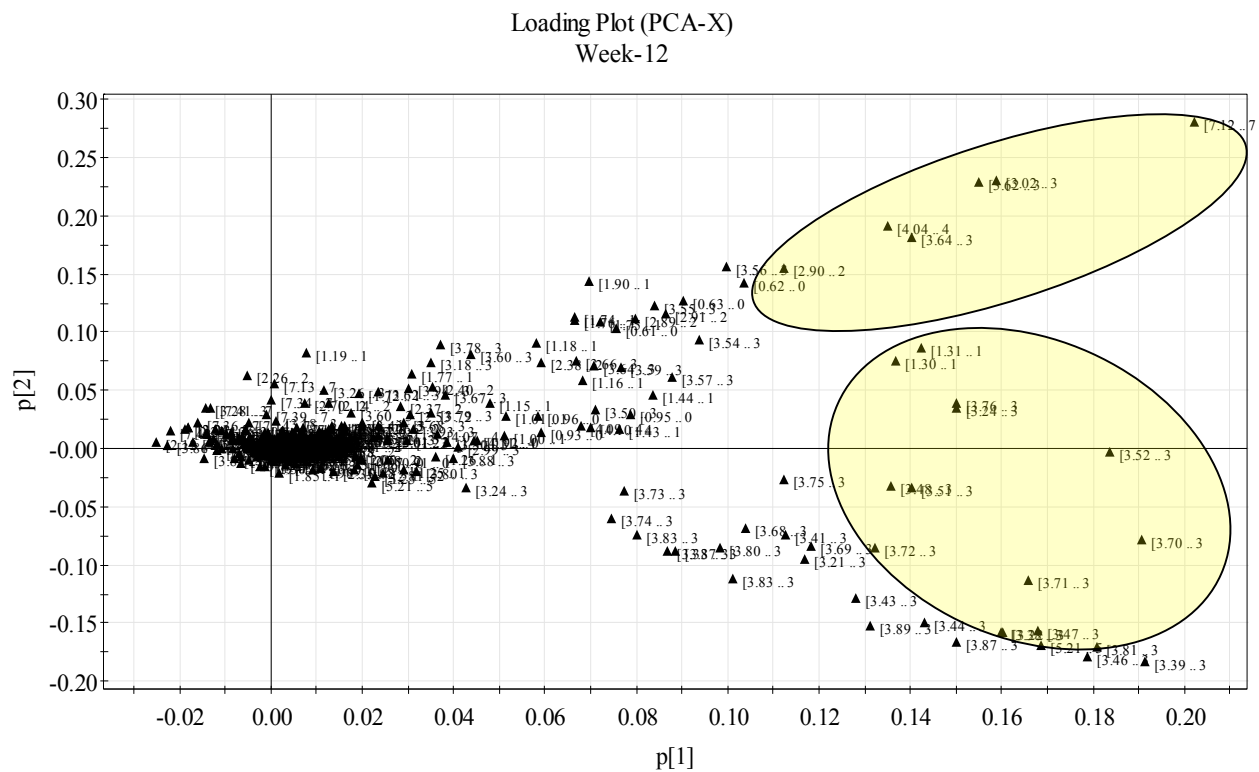
Abbreviations: PCA, principal component analysis.

Note: This figure shows the corresponding PCA-X loading plot for **Figure 6-6**. The loading plot provides information regarding the part of the spectrum that was different or common between the two groups. The dense, dark area represents common metabolites (part of spectrum) that are shared between the two groups while the isolated plots indicate the part of the spectrum that responsible for the difference (yellow circles).

FIGURE 6-9. PLS score plot for correlation between metabolomics profile and inflammatory markers at week-12.
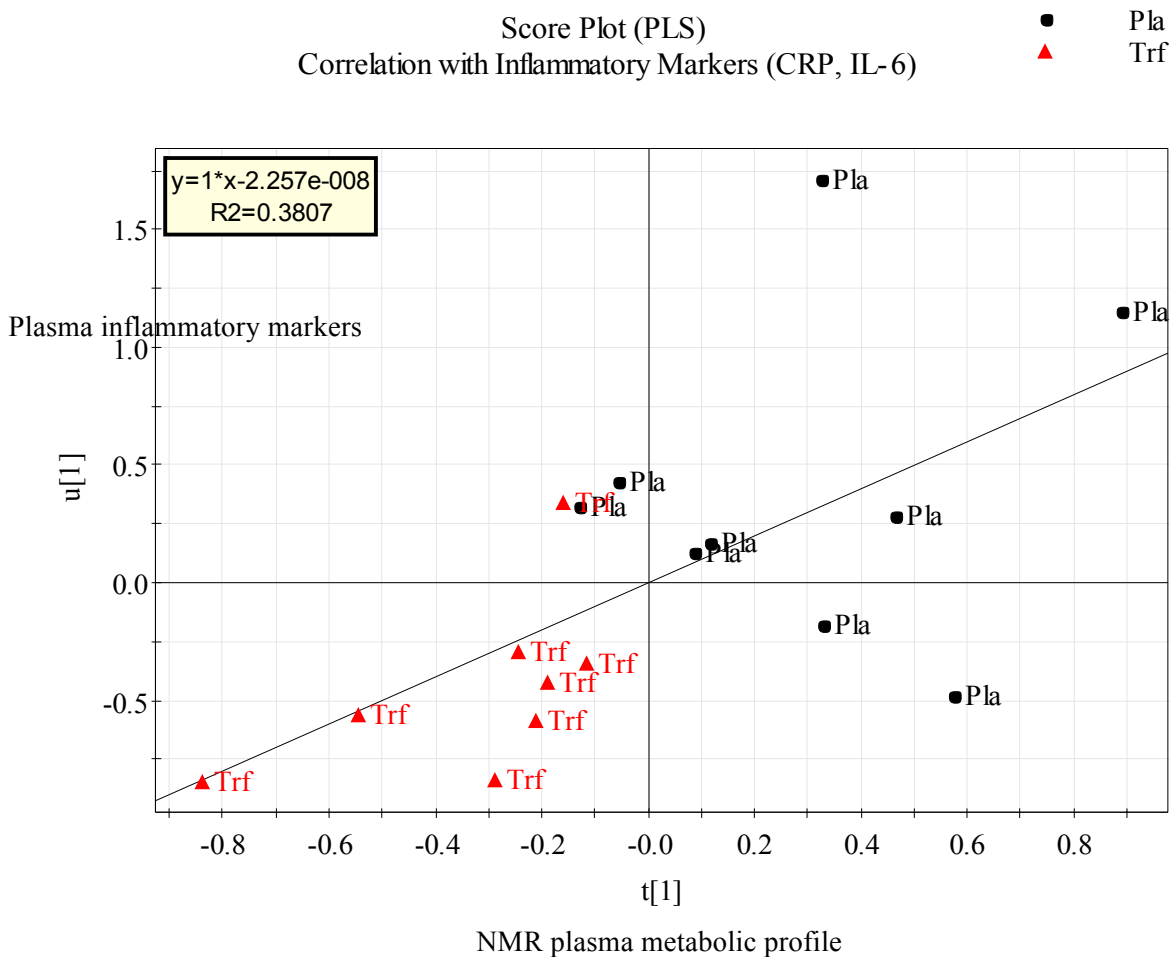
Abbreviations: PLS, partial least square; PLA, placebo; TRF, tocotrienol-rich fraction; CRP, C-reactive protein; IL-6, interleukin-6.

Note: This figure shows PLS score plot depicting correlation between metabolomics profile (x-axis) and inflammatory markers (CRP, IL-6) (y-axis) of the two groups at week-12. There was a moderate correlation (R^2=0.38) between metabolomics profile and inflammatory markers. This result indicates that there are some metabolites in the plasma sample that are correlated with the inflammatory markers.

FIGURE 6-10. PLS score plot for correlation between metabolomics profile and oxidative status biomarkers (TAP, MDA) at week-12.

Abbreviations: PLS, partial least square; PLA, placebo; TRF, tocotrienol-rich fraction; TAP, total antioxidant power; MDA, malondialdehyde.

Note: This figure shows PLS score plot depicting correlation between metabolomics profile (x-axis) and total antioxidant capacity (y-axis) of the two groups at week-12. There was a moderate correlation (R^2=0.26) between metabolomics profile and TAP. This result indicates that there are some metabolites in the plasma sample that are correlated with the TAP.

FIGURE 6-11. PLS score plot for correlation between metabolomics profile and plasma HDLC at week-12
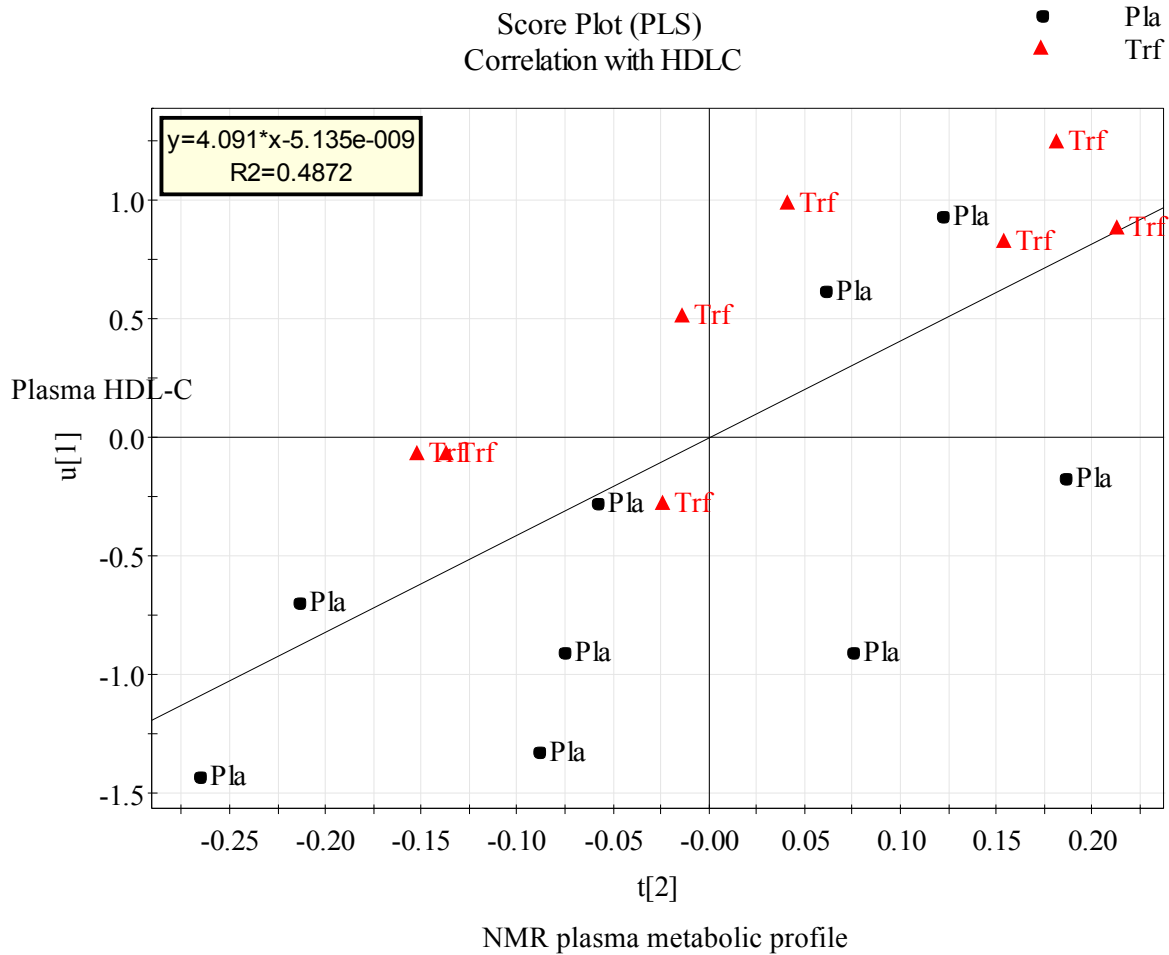
Abbreviations: PLS, partial least square; PLA, placebo; TRF, tocotrienol-rich fraction; HDL-C, high-density lipoprotein cholesterol.

Note: This figure shows PLS score plot depicting correlation between metabolomics profile (x-axis) and total antioxidant capacity (y-axis) of the two groups at week-12. There was a moderate correlation (R^2=0.49) between metabolomics profile and HDL-C. This result indicates that there are some metabolites in the plasma sample that are correlated with the HDL-C.

FIGURE 6-12. PLS score plot for correlation between metabolomics profile and plasma lipid profiles (TC, TAG, HDLC) at week-12
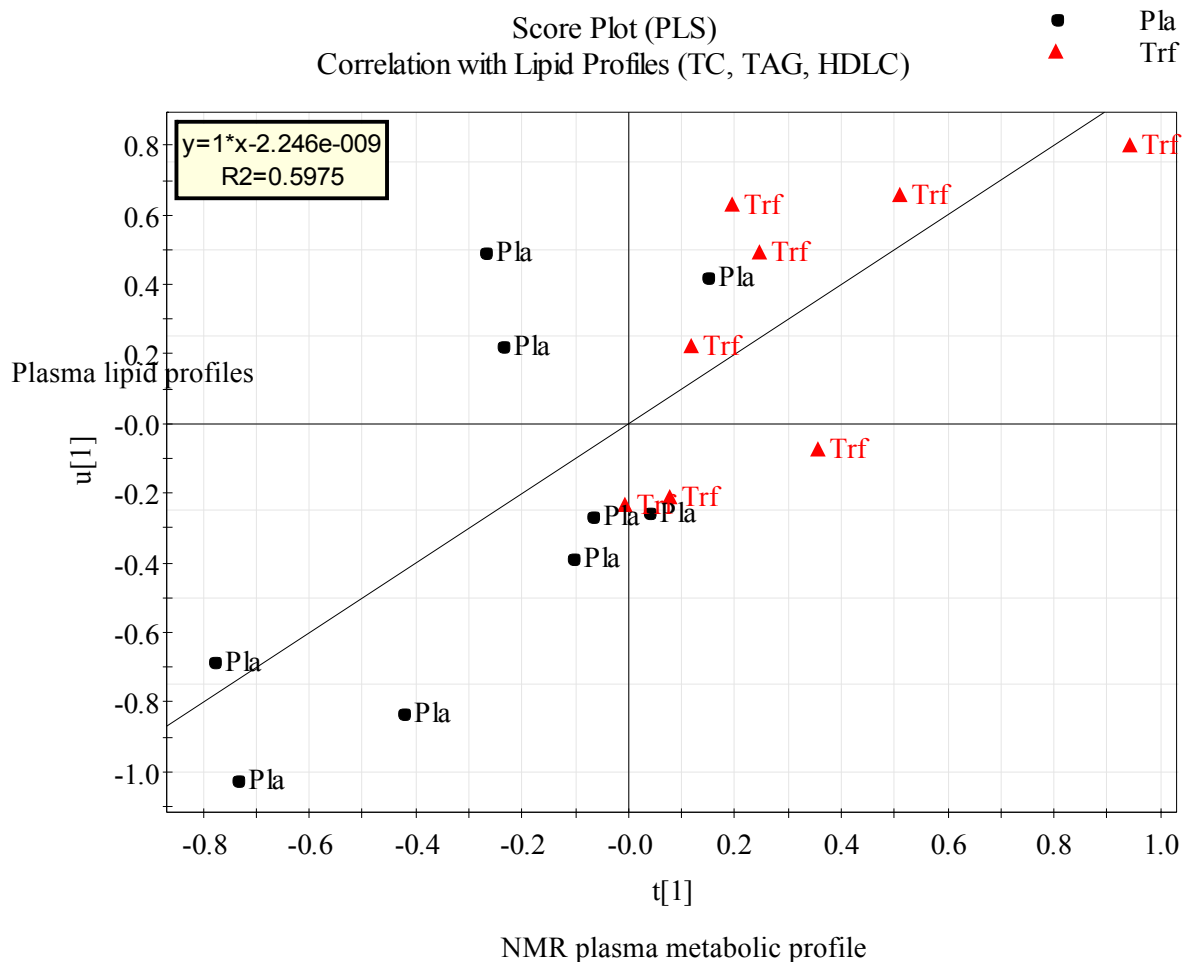
Abbreviations: PLS, partial least square; PLA, placebo; TRF, tocotrienol-rich fraction; TC, total cholesterol; TAG, triacylglycerols; HDL-C, high-density lipoprotein cholesterol.

Note: This figure shows PLS score plot depicting correlation between metabolomics profile (x-axis) and plasma lipid profiles (TC, TAG, and HDL-C) (y-axis) of the two groups at week-12. There was a moderate correlation (R^2=0.60) between metabolomics profile and lipid profiles. This result indicates that there are some metabolites in the plasma sample that are correlated with the lipid profiles.
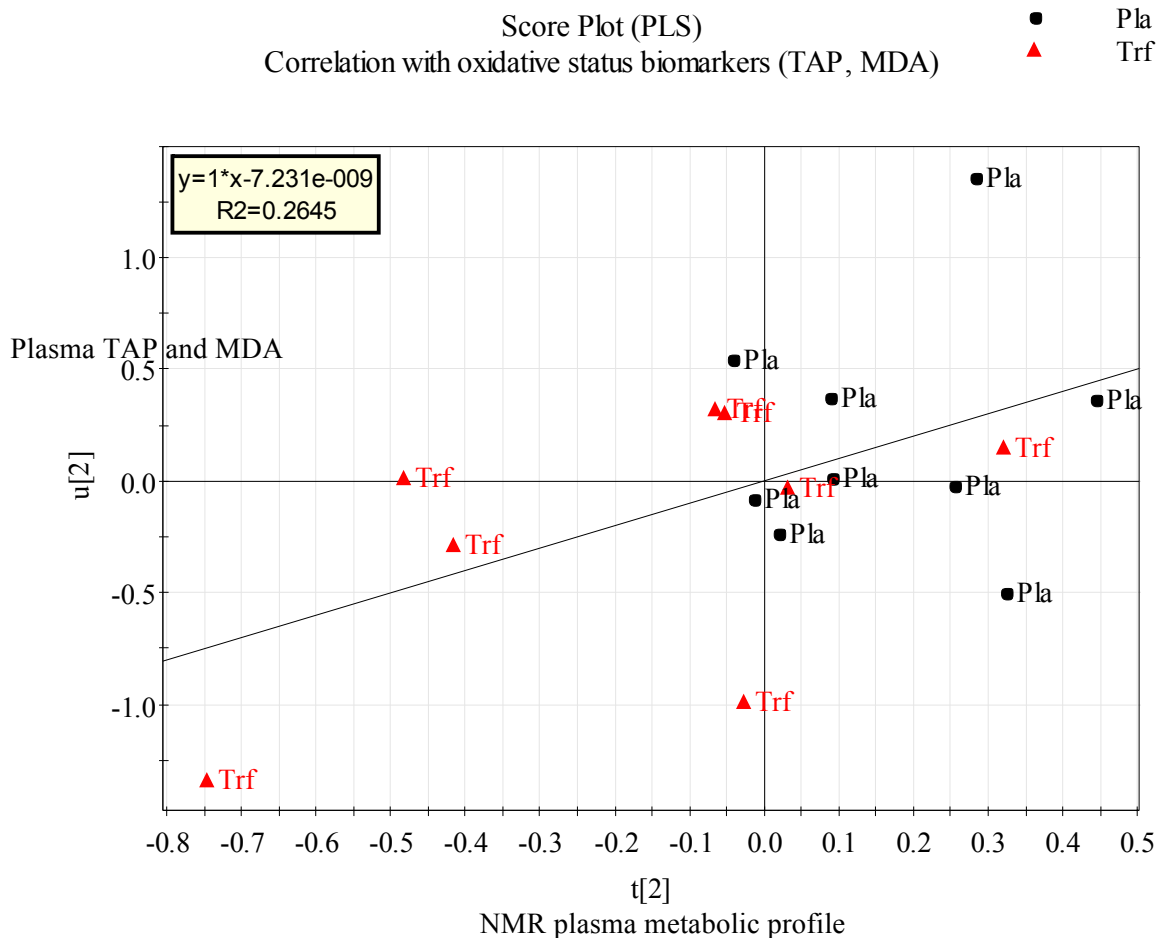
FIGURE 6-13. PLS score plot for correlation between metabolomics profile and serum albumin at week-12.

Abbreviations: PLS, partial least square; PLA, placebo; TRF, tocotrienol-rich fraction

Note: This figure shows PLS score plot depicting correlation between metabolomics profile (x-axis) and serum albumin levels (as a marker of nutritional status) (y-axis) of the two groups at week-12. There was a moderate correlation (R^2=0.65) between metabolomics profile and lipid profiles. This result indicates that there are some metabolites in the plasma sample that are correlated with the serum albumin levels.

CHAPTER VII

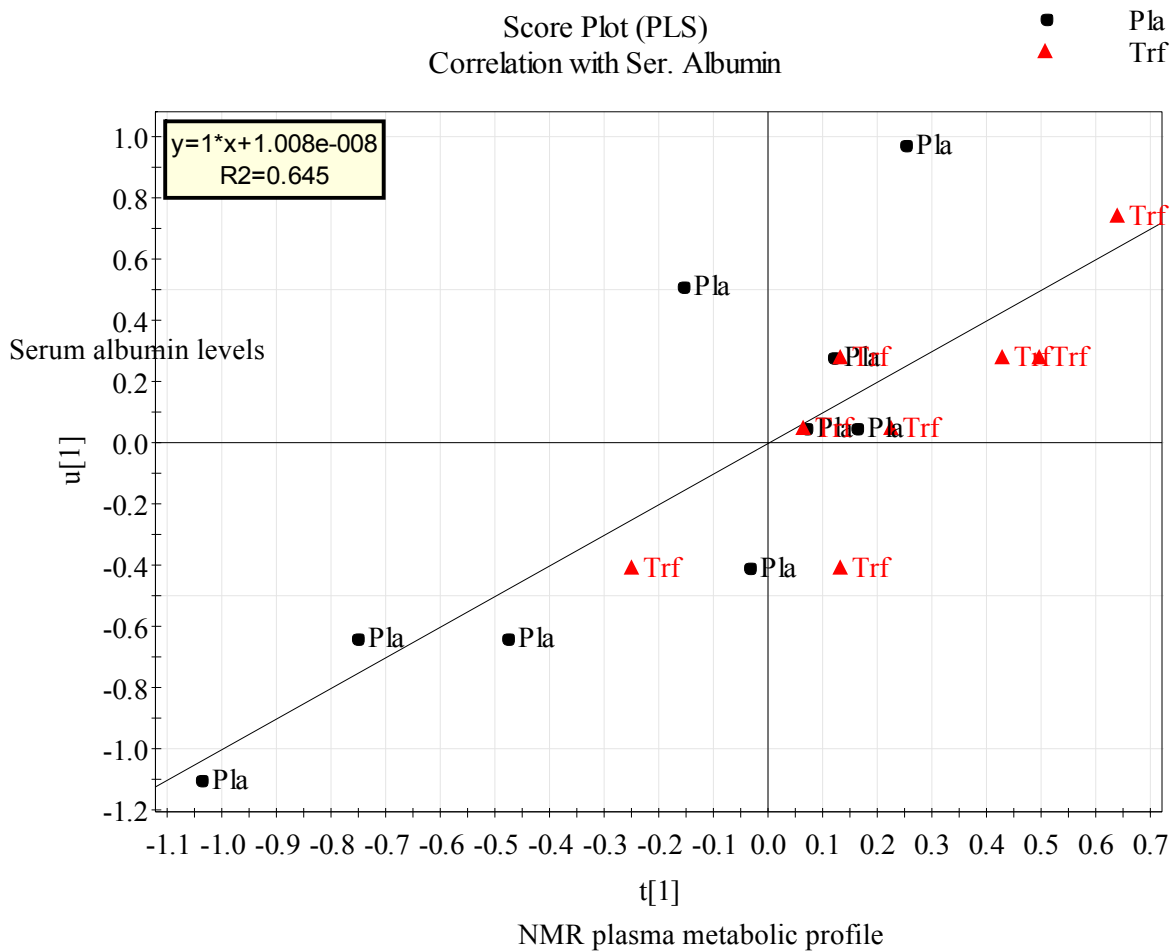## CONCLUSION AND RECOMMENDATIONS

Conclusion

The aim of the first study was to evaluate the effects of omega-3 combined with hydrolyzed protein supplementation on the nutritional status, inflammatory markers and lipid profiles in chronic HD patients. The study was designed in such way that supplementation intake can be directly monitored in real-time in order to maximize the compliance given the fact that our study population was mainly comprised of low-income and low-literacy group which often associated with poor compliance. We found that omega-3 group had significant improvement in LDLC/HDLC ratio while TAG was marginally improved. However, no significant different were seen in nutritional and inflammatory parameters between placebo and omega-3 groups after 6-months of supplementation. While compliance was excellent in patients who attended the treatment session, significant numbers of patients missed the treatment due to hospitalization, thus also missed their supplementation – this potentially had an impact on the overall study results. Results from this study support the hypothesis that the combination of omega-3 and protein supplementation improve lipid profiles but fail to show significant effect on inflammatory and nutritional indicators.

Following a successful 'directly observed treatment' approach in omega-3 study, a second study was administered using vitamin E tocotrienols (TRF), a nutritional agent that exhibit antioxidant, anti-inflammatory with a potential lipid altering effects in the same cohort of the patients. Given the significant comorbidities experienced by these patients that resulted in significant hospitalization and missed dialysis treatment in the first study, therefore additional

steps were taken in order to improve compliance in administering the supplements. Patients were provided with capsule organizer for supplement supply during non-dialysis days and compliance were monitored via pill counting method and 'directly observed treatment'. Additional monitoring on dietary changes (through 24-hr diet recalls) was also implemented. The results showed that TRF supplemented group had significant improvement in lipid profiles as indicated by a lower TAG, and higher HDLC at week-12 and week-16 of the study when compared to the placebo group. Interestingly, TRF group also exhibit less total hospitalization days as compared to the placebo group. However, TRF supplementation did not significantly impact any nutritional, inflammatory and oxidative status biomarkers.

Following the unexpected outcomes in lipids, we sought to investigate further on several key components of lipid metabolism namely, CETP and ApoA1. We found that the change in TAG and HDLC seen in TRF group were related to a higher ApoA1 concentration and lower CETP activity, indicating a potential increase in HDL particle in TRF group. As a conclusion, further study in a larger cohort of patients (multi-center) is needed to confirm this observation. Furthermore, an increased in sample size will allow sub-group analyses to elucidate the cofounding effect of various medications and underlying diseases in this population.

Given the limitation of the traditional biomarkers to provide information on a global impact of TRF supplementation, the metabolomics approach was used to elucidate whether the TRF supplementation lead to overall changes in patients' metabolomics profile and whether the observed changes in plasma lipids correlates with their metabolomics profile. Based on the multivariate data analysis (PCA, PLS-DA), it was shown that metabolomics profiles of TRF group were different from placebo group at week-12 of the intervention. Moreover, metabolomics profile in both TRF and placebo group was correlated with inflammatory markers

and lipid profiles suggesting that some plasma metabolite could predict/ responsible for the changes in lipid profiles and inflammatory markers. Preliminary results from metabolites identification and quantification revealed that TRF supplementation triggered metabolic changes leading to either reduction or increased in several metabolites that have been reported otherwise. These include reduction in 2-Hydroxyglutarate, 3-Hydroxy-3-methylglutarte, Betaine, Cadaverine, Glycerate, Mandelate, Proline and 2-Hydroxy-3-methylvalerate. On the other hand, metabolites that were significantly higher in TRF group include Citrate, N-acetylaspartate, N-acetylcysteine and Ribose. Further study is needed to validate the respective metabolites that contribute to the different metabolomics profile in TRF group with biochemistry test.

Recommendations and future directions

The first study added to the known knowledge regarding omega-3 fatty acids effects on lipid profiles, particularly in reducing TAG. However, additional study in a larger scale is needed to confirm omega-3 fatty acids effects on inflammatory markers since literature in this area is still ambiguous, partly due to high variability in the inflammatory markers. Such study should also include measurement of plasma fatty acids together with dietary records to confirm compliance.

The second study has provided exploratory data on the benefit of tocotrienol supplementation particularly in improving lipid profiles. However, given the shortcomings of the study and the fact that it was conducted in a homogenous population, the impact of TRF in a diverse dialysis population is unknown and yet to be recommended. Therefore, the precedent study should consider a multi-center approach to confirm the current observation, improve the study's power and enable subgroup analysis (e.g. diabetic *vs* non-diabetic; statin *vs* non-statin users). Future study should incorporate more robust measurements for lipids and inflammatory

parameters (e.g. LCAT, lipoprotein particle size, cytokines and pro-inflammatory eicosanoids) to understand the mechanism of action. Moreover, a dose escalation study should also be considered to identify optimum dosage and duration to achieve the target lipid and inflammatory parameters as well as to recognize adverse effects (if there is any, especially at higher dosage).

It would also be of interest to investigate synergistic effects of omega-3 and TT in this population. Finally, intervention aimed at improving inflammatory biomarkers would require a large study population and extensive funding due to a substantial variability in these biomarkers. However, with the use of metabolomics approach, overall understanding of cardiovascular disease states beyond traditional biomarkers will facilitate discovery of novel biomarkers and metabolic pathways.

APPENDIX A: 24-HOUR DIETARY RECALL FORM

Name: [   ]   Date: [   ]

Time / Meals	Food eaten	Quantity / portion size / amount/ numbers	Remarks (if appropriate – add additional information) (e.g. how prepared?, brand name?, added cream/sugar, where eaten?)

APPENDIX B: EXAMPLE OF DIETARY ANALYSIS PERFORMED USING NUTRITIONIST PRO

📝 Menu Template Nutrient Analysis

Total Days: 1
Total Foods: 16

Avg. Daily Kcals: 2186.680
Name: 20b

Nutrient	Value	Unit	Goal	%	Nutrient	Value	Unit	Goal	%
Weight	1914.2	g			Phosphorus	1365.460	mg	1000.000	137 %
Kilocalories	2186.7	kcal	2000.0	109 %	Iodine			150.000	
Protein	111.4	g	50.0	223 %	Magnesium	218.531	mg	400.000	55 %
Carbohydrate	224.9	g	300.0	75 %	Zinc	10.774	mg	15.000	72 %
Fat, Total	95.0	g	65.0	146 %	Copper	0.847	mg	2.000	42 %
Alcohol	0.0	g			Manganese	1.340	mg	2.000	67 %
Cholesterol	719.9	mg	300.0	240 %	Selenium	139.567	µg	70.000	199 %
Saturated Fat	27.9	g	20.0	140 %	Fluoride	265.774	µg		
Monounsaturated Fat	37.1	g			Chromium	0.074	mg	0.120	62 %
Polyunsaturated Fat	21.1	g			Molybdenum	185.911	µg	75.000	248 %
MFA 18:1, Oleic	32.4	g			Dietary Fiber, Total	12.958	g	25.000	52 %
PFA 18:2, Linoleic	14.6	g			Soluble Fiber	0.000	g		
PFA 18:3, Linolenic	1.5	g			Insoluble Fiber	0.000	g		
PFA 20:5, EPA	0.0	g			Crude Fiber	4.369	g		
PFA 22:6, DHA	0.0	g			Sugar, Total	21.292	g		
Trans Fatty Acid	0.0	g			Glucose	4.864	g		
Sodium	4279.4	mg	2400.0	178 %	Galactose	0.000	g		
Potassium	3377.7	mg	3500.0	97 %	Fructose	4.314	g		
Vitamin A (RE)	651.8	RE			Sucrose	8.605	g		
Vitamin A (IU)	3773.9	IU	5000.0	75 %	Lactose	0.000	g		
Vitamin A (RAE)	505.6	µg			Maltose	0.495	g		
Beta-Carotene	1680.4	µg			Sugar Alcohol				
Alpha-Carotene	274.5	µg			Other Carbohydrate				
Lutein (+ Zeaxanthin)	1943.0	µg			Tryptophan	1239.329	mg		
Beta-Cryptoxanthin	0.0	µg			Threonine	4535.460	mg		
Lycopene	1210.0	µg			Isoleucine	4730.796	mg		
Vitamin C	490.1	mg	60.0	817 %	Leucine	8138.050	mg		
Calcium	561.7	mg	1000.0	56 %	Lysine	8166.761	mg		
Iron	14.6	mg	18.0	81 %	Methionine	2613.008	mg		
Vitamin D (ug)	1.2	µg	10.0	12 %	Cystine	1531.321	mg		
Vitamin D (IU)	49.6	IU	400.0	12 %	Phenylalanine	4439.847	mg		
Vitamin E (mg)	6.1	mg	20.0	30 %	Tyrosine	3507.970	mg		
Vitamin E (IU)	9.1	IU	30.0	30 %	Valine	5309.381	mg		
Alpha-Tocopherol	1.4	mg			Arginine	6189.328	mg		
Thiamin	3.9	mg	1.5	263 %	Histidine	3439.898	mg		
Riboflavin	2.2	mg	1.7	127 %	Alanine	5737.192	mg		
Niacin	24.5	mg	20.0	122 %	Aspartic Acid	9574.290	mg		
Pyridoxine (Vitamin B6)	1.7	mg	2.0	86 %	Glutamic Acid	16841.620	mg		
Folate (Total)	245.2	µg	400.0	61 %	Glycine	4990.013	mg		
Folate (DFE)	259.9	µg			Proline	4971.783	mg		
Cobalamin (Vitamin B12)	3.7	µg	6.0	62 %	Serine	4830.367	mg		
Biotin	24.1	µg	300.0	8 %	Moisture	972.189	g		
Pantothenic Acid	4.2	mg	10.0	42 %	Ash	19.200	g		
Vitamin K	27.7	µg	80.0	35 %	Caffeine	137.460	mg		

Nutrient Goal Template
DAILY VALUES/RDI - ADULT

Analyzed by
Zul

Percentage of Kcals
Protein	20.2%
Carbohydrate	40.9%
Fat, total	0.389
Alcohol	0.0%

Exchanges
Bread/Starch	6
Fat	6.50
Fruit	6.50
Meat-High Fat	0.50
Meat-Medium Fat	11.00
Other Carbohydrate	0.50
Vegetable	4.00

Tuesday, October 01, 2013 — Page — *First DataBank Nutritionist Pro*

Note: Example of the output from analysis of energy and nutrients intake from one subject using Nutritionist Pro.

APPENDIX C: COMPLIANCE MONITORING FORM

Name	Shift	Group	Pt present		Capsules given		Container given		Container returned		Initials
1. AI01	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
2. DB02	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
3. FD03	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
4. HJ04	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
5. HJ05	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
6. LT06	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
7. MJ07	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
8. OC08	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
9. AR09	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
10. RL10	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
11. RM11	1	B	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
12. RE12	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
13. SJ13	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	
14. TS14	1	A	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	☐ Yes	☐ No	

APPENDIX D: QUANTITATIVE ANALYSIS OF TOCOTRIENOL LEVELS BY EXTERNAL LABORATORY

Introduction
In this study HPLC was used for separation, determination and quantification of individual tocotrienol levels in human plasma after the oral administration of TRF (fasting plasma samples).

Materials and Methods
Sample Preparation
Human plasma samples were stored at -80oC until use. For extraction, 150 µL of plasma was added to a glass tube with screw cap and kept on ice. To this 2 mL of 1% ascorbic acid in ethanol (w/v), 900µL of deionized water, 25 µL of 1% BHT (w/v) in ethanol and 2 mL of hexane were added. The tubes were screw capped and contents were mixed by hand inversion followed by centrifugation at 1000rpm for 4 min. After the phase separation, supernatant hexane layer was taken into a new labeled test tube which consists of lipophilic tocopherols and tocotrienols. The extraction was repeated by adding 2 mL of hexane and mixing it by hand inversion followed by centrifuigation at 1000 rpm for 4 min. The supernatant hexane layer was added to the previous extract and dried under vacuum with centrifugal evaporator. The residue obtained is suspended in 200 µL of mobile phase and placed in deactivated glass auto-injector insert for analysis.

Chromatographic conditions
Analysis of tocotrienols was performed according to the method described below. An aliquot of 20 µL was injected into HPLC system (Dionex) coupled to Dionex RF 2000 floerescene detector. The pump was run at a flow rate of 1.8ml/min and the mobile phase used was composed of methanol, isopropanol, NaClO4 (85.10.5). Mobile phase was filtered through 0.2 µm nylon membrane before use. The total run time was 18 min and chromatographic separation was carried out by a phenomenex reversed phase column (5µm, 4.6mm x 250mm). The eluate was monitored for peaks by fluorescence detector set at excitation wavelength of 296nm and emission wavelength of 330nm. Calibrations were linear for the determination of α-tocopherol and γ-tocotrienol.

Standard curve and linearity
Stock solution of γ-tocotrienol and α-tocopherol (1mM) were prepared in mobile phase. For standard curve, γ-tocotrienol was diluted by mobile phase to give concentration of 0.05, 0.1, 0.25, 0.5, 1 and 2 µM and α-tocopherol was diluted to 0.1, 0.25, 0.5, 1, 2.5 and 5 µM. The retention time for γ-tocotrienol and α-tocopherol were found to be 7.1min and 14.2 min (appox.).

Results

All the standard and test samples were run for analysis and images are shown below. Individual tocotrienols were shown in following Table.

Sample #				γ-tocotrienol		α-tocotrienol		δ-tocotrienol		α-tocopherol	
				Peak area	Conc. (ng in 100µL)	Peak area	Conc. (ng in 100µL)	Peak area	Conc. (ng in 100µL)	Peak area	Conc. (ng in 100µL)
Placebo 1				ND	ND	ND	ND	ND	ND	1921577	157
Placebo 2				ND	ND	ND	ND	ND	ND	1576941	103
Placebo 3				ND	ND	ND	ND	ND	ND	1172591	100.2
Placebo 4				ND	ND	ND	ND	ND	ND	1137831	52.7
Placebo 5				12114	0.24	ND	ND	ND	ND	635672	209
TRF1				144047	0.14	ND	ND	ND	ND	1576941	134.6
TRF2				197457	0.4	ND	ND	ND	ND	3019545	226.5
TRF3				ND	ND	ND	ND	ND	ND	2273772	203.9
TRF4				ND	ND	ND	ND	ND	ND	3548170	270.9
TRF5	ND	ND	ND	ND	ND	ND		3225131		269.2	

Standard curve
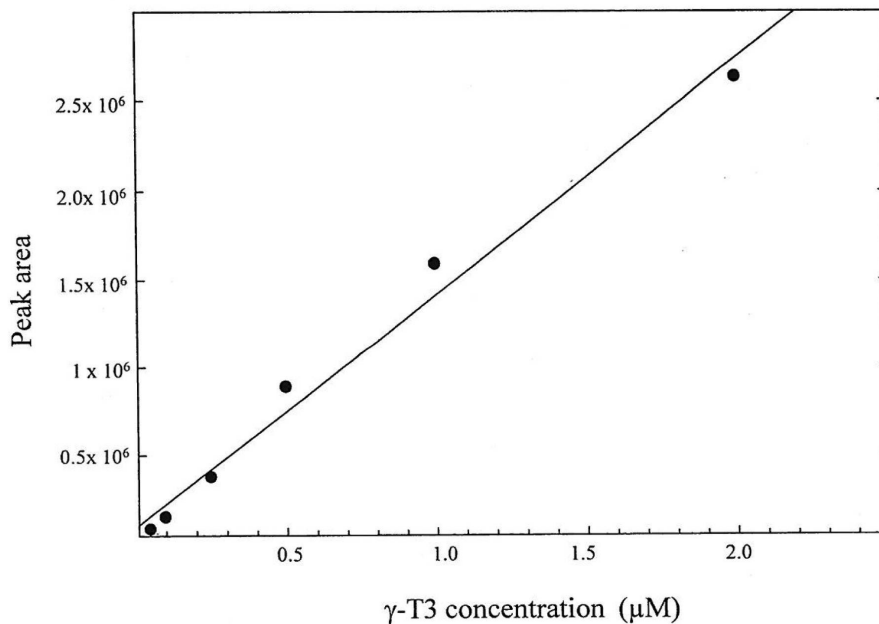
Fig 1:
Calibration curve for γ-Tocotrienol in methanol-isopropanol-sodium perchlorate (85-10-5).
r² , 0.9844
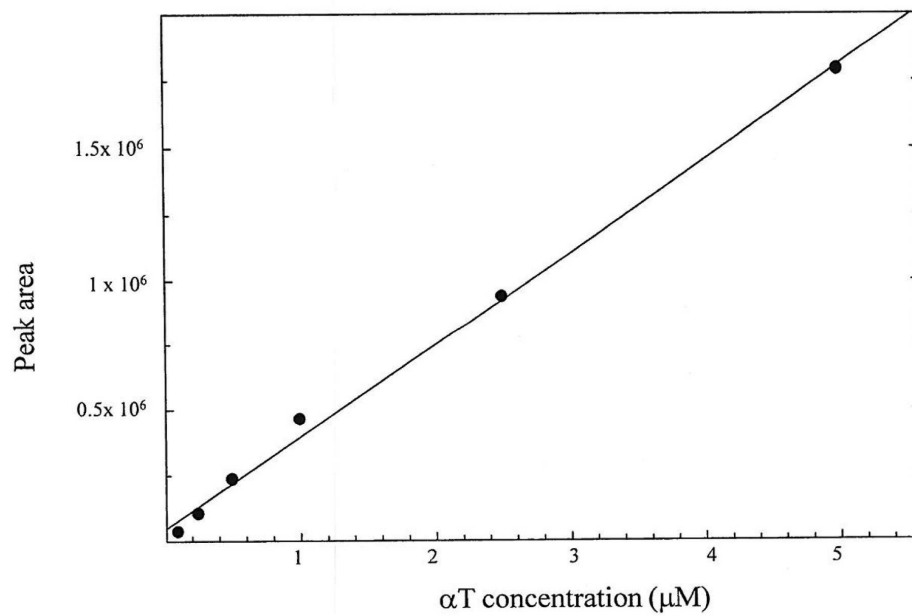
Fig 2:
Calibration curve for α-Tocopherol in methanol-isopropanol-sodium perchlorate (85-10-5).
r^2 , 0.9964

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ABSTRACT

MULTIFACETED NUTRITIONAL INTERVENTION IN HEMODIALYSIS PATIENTS

by

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Mortality rate in patients undergoing chronic hemodialysis (HD) in the United States remain unacceptably high despite improvement in dialysis technology. Cardiovascular disease (CVD) account for more than 50% of the premature death in this population. Evidences indicate that the accelerated CVD are attributed to by malnutrition, inflammation, dyslipidemia and oxidative stress. Dietary intervention using nutrients with antioxidant, anti-inflammatory, and potential lipid altering properties to correct the aforementioned problems remain inconclusive. Furthermore, such nutritional intervention trial is often hampered by poor compliance related to medical and socioeconomic barriers. Therefore, a series of randomized, double-blind, placebo-controlled, parallel trials were undertaken to document the technical feasibility of 'directly observed treatment' and the global impact of several nutrients namely omega-3 and vitamin E tocotrienols on nutritional and oxidative indicators, inflammatory markers and lipid profiles in a cohort of chronic HD patients.

The objective of the first study was to evaluate the impact of omega-3 plus liquid protein supplement on serum albumin, plasma lipids and other indicator of nutrition and inflammation.

markers. The study recruited 63 subjects and they were randomized into placebo+protein (n=32) and omega-3+protein (n=31) groups. The two intervention groups received 30mL of a liquid protein plus 2.4g omega-3 or placebo, three times per week after their routine dialysis session for 6 months. Directly observed nutritional supplement resulted in significant improvement in the LDLC/HDLC ratio in the omega-3 group as compared to the placebo group (P=0.043). In the omega-3 group, serum albumin was also marginally higher after 6 months as compared to the baseline (P=0.07). The observed increase in CRP levels in the placebo group over 6 months was not apparent in the omega-3 group, although there was no significant difference between groups. NFκB, MIS, nPNA, BMI and hemoglobin were unaffected by the intervention. Therefore, it is conclude that 'directly observed treatment' with an omega-3 based supplement (as opposed to a pure protein supplement) showed beneficial effects on lipid profile, and CRP levels. Further studies using a combination of outpatient and inpatient 'directly observed treatment' is warranted.

Given a proven feasibility of directly observed treatment in the first study, we conducted a second study using tocotrienol rich fractions (TRF) by incorporating the same design to maximize compliance but with additional take home supplements. Vitamin E tocotrienols have been reported to confer anti-inflammatory, antioxidant and a potential of lipid altering benefits *in vitro*, *in vivo* and in some other clinical population. However, the impact of this nutrient in HD population is unknown. Subjects were provided daily with capsules containing either vitamin E tocotrienol-rich fraction (TRF) (180 mg tocotrienols, 40 mg tocopherols) or placebo (0.48 mg tocotrienols, 0.88 mg tocopherols). For the results, TRF supplementation did not impact any nutritional, inflammatory, or oxidative status biomarkers over time when compared with the baseline within the group (one-way repeated measures analysis of variance) or when compared

with the placebo group at a particular time point (independent t-test). However, the TRF supplemented group showed improvement in lipid profiles after 12 and 16 weeks of intervention when compared with placebo at the respective time points. Normalized plasma triacylglycerols (cf baseline) in the TRF group was reduced by 33 mg/dL (P=0.032) and 36 mg/dL (P=0.072) after 12 and 16 weeks of intervention but no significant improvement was seen in the placebo group. Similarly, normalized plasma high-density lipoprotein cholesterol was higher (P<0,05) in the TRF group as compared with placebo at both week 12 and week 16. The changes in the TRF group at week 12 and week 16 were associated with higher plasma apolipoprotein A1 concentration (P<0.02) and lower cholesteryl-ester transfer protein activity (P<0.001). As a conclusion, TRF supplementation improved lipid profiles in the study of maintenance HD patients. A multi-centered trial is warranted to confirm these observations.

Finally, following a positive impact of TRF supplementation on lipid profiles, we undertook a metabolomics approach to investigate whether the TRF supplementation lead to overall changes in patients' metabolomics profile and whether the observed changes in plasma lipids correlates with their metabolomics profile. Based on the principal component analysis (PCA), there was a separation pattern between the TRF and placebo groups at week-12. After applying partial least square-discriminant analysis (PLS-DA), there was a clear separation between the two groups indicating different metabolomics profiles. In addition, metabolomics profile in both TRF and placebo group was correlated with inflammatory markers and lipid profiles suggesting that some plasma metabolite could predict/ responsible for the changes in lipid profiles and inflammatory markers.

AUTOBIOGRAPHICAL STATEMENT

Mr. Zulfitri A. Mat Daud, MS, RD received his undergraduate education in Dietetics from Universiti Sains Malaysia (USM), Kubang Kerian, Kelantan, Malaysia. Soon after, he joined Universiti Putra Malaysia (UPM) as a tutor and subsequently pursued his M.S. degree from National University of Malaysia (UKM), Kuala Lumpur, Malaysia. Through his M.S. research, he gained his research interest in renal nutrition and metabolism. His hard work and diligence in his M.S. research being paid off when he won several awards from free paper presentations at the local and international levels namely; 1) best oral communication (dietitian program) at 14th International Congress on Nutrition and Metabolism in Renal Disease, Marseilles, France, 2) second place for oral presentation at 22nd MSN Annual Seminar in Nephrology, Johor Baharu, Malaysia, 3) second place for poster presentation at 6th National Symposium on Health Sciences, Kuala Lumpur, Malaysia, and 4) best poster prize at 3rd PENSMA Scientific Meeting. He also had been awarded "Best Post-Graduate Prize" in 2009 from Nutrition Society of Malaysia for a distinguished MS thesis. Mr. Daud received a precious opportunity to further establish his career in academia when he was awarded a scholarship from the Malaysian Ministry of Higher Education to pursue his advance degrees at Wayne State University (WSU), Detroit, Michigan. In WSU, he obtained a second MS degree specializing in lipid metabolism and successfully received a credential as a Registered Dietitian (RD) from the US Commission of Dietetic Registration. Mr. Daud is currently continuing his graduate degree specializing in renal nutrition towards accomplishment of PhD degree requirement at WSU. Mr. Daud is also actively contributing to the community by volunteering as a board member in Southeastern Michigan Dietetic Association (SEMDA) and participating in a student run wellness clinic (as a nutrition counselor) cater for uninsured patients around metro Detroit area.