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Effects Of Tocotrienol Rich Fractions On Lipid Profiles In Hemodialysis Patients

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EFFECTS OF TOCOTRIENOL RICH FRACTIONS ON LIPID PROFILES IN HEMODIALYSIS PATIENTS

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DEDICATION

This thesis is dedicated to:

Rafik Hanna and Suhaila Nseir
My parents who worked hard for us

Fadi Hanna
My brother

My good loyal friends
Who stood by me in my darker days

To all and everyone who helped me achieve my goal in life

To all and everyone who did good to mankind and avoided negativity

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Chronic Kidney Disease

Chronic kidney disease (CKD) is a long-standing, progressive (irreversible) deterioration of renal function. It affects about 10-15% of adults in the Western world. Based on the National Kidney Foundation of Kidney Disease, there are about 26 million individuals with CKD in the USA [1-3].

Symptoms of CKD develop slowly and include anorexia, nausea, vomiting, stomatitis, dyspepsia, nocturia, lassitude, fatigue, pruritus, decreased mental acuity, muscle twitches and cramps, water retention, under nutrition, gastrointestinal ulceration and bleeding, peripheral neuropathies, and seizures. CKD should be distinguished from acute kidney failure which tends to be most acute and reversible [4]. However, if the causes of the acute failure persist or if it is repeated, the kidneys will deteriorate. Repeated acute kidney failure may lead to CKD.

Diagnostic Criteria of CKD

The diagnosis criteria were established by Kidney Disease Outcome Quality Initiative K/DQQI in 2002 and The National Institute of Diabetes and Digestive and Kidney Diseases. Criteria of CKD include either evidence of progressive kidney damage or an irreversible pathologic abnormality that persists for at least 3 months, with or without reduction in glomerular filtration rate (GFR), and proved pathologically or clinically; or persistent reduction for three months in GFR to < 60 mL/min/1.73 m.^{2} Normal GFR ranges from 90 - 120 ml/min/1.73 m^2

The diagnosis is made by laboratory and/or by confirming anatomical and histological damages. The reduction of GFR of 60 to 89, 30 to 59, and 15 to 29 ml/min/1.73 m^2 defines stages 2,3, and 4 of CKD; respectively [3, 5]. A less than 15 mL/min/1.73 m^2 defines end stage renal

disease or ESRD and is an indication for dialysis. Once the GFR of an individual reaches that of ESRD, the treatment will include a form of dialysis or kidney transplant [3, 4].

Causes of CKD

The two most common causes of CKD in the Unites States are poorly treated chronic diabetes mellitus and poorly regulated chronic hypertension [1, 3-5]. Age remains an independent risk factor for CKD and CKD is more prevalent among African Americans and Native Americans because of diabetes and metabolic syndrome [2, 3].

Clinical textbooks and literatures list other causes of CKD [3, 4, 6]. Autoimmune disorders such as systemic lupus erythematosus are major causes because the kidneys are highly sensitive to immune reaction and antibodies [2, 7]. Glomerulonephritis is an auto immune disease and often associated with proteinuria and/or hematuria that may be linked to autoimmunity or microbial infection. Chronic nephrotic syndrome is associated with albuminuria [7].

Cardiovascular diseases (CVD) may be caused by reduction of blood supply (ischemia) or to thrombo-embolic effect can lead to CKD. CVD includes hypertension, infarction, cardiovascular failure, chronic congestive heart failure (CHF), viral and bacterial endocarditic (often associated with auto-antibody to the kidneys and bad oral hygiene), and rarely aneurysm in the renal arteries.

Metabolic diseases includes endocrinopathies such as hyperparathyroidism (may be a cause or a consequence of CKD), hyper-aldosteronism (which leads to hypertension), diabetes mellitus and metabolic acidosis. Injury or trauma that has not been properly treated could lead to permanent damage to both kidneys [4].

Kidney stones, when poorly treated, may lead to structural damages. Kidney stones may or may not be calcified (Non-calcified stones may be missed by regular X-rays). Gout (uric acid), oxalates, and other compounds may cause kidney stones and kidney damages. Repeated renal infections or reflex infection (backward from the bladder, when associated with ascending urinary retention that has not been treated) are common causes of CKD, notably in economically disadvantaged communities. Rarely, parasites may damage the kidneys (immune mediated, recurrence of hemolytic or bleeding, or direct invasion); these parasites include malaria and Schistozomiasis [4].

Chronic obstructive uropathy may be caused by non-infectious factors. It damages the kidneys by reflex or backward flow of urine that may be caused by an untreated and undetected tumor (the tumor may be benign otherwise, undetected stones or other obstructive causes). This formerly fell into post-renal conditions that cause chronic accumulation/retention of urine in the kidneys and damage the kidneys. Obstruction may cause damage to one kidney or both kidneys. If one kidney is damaged, chronic alteration of blood pressure and blood homeostasis may affect the other one [2-4].

Medications, including certain anti-inflammatory and pain medications, and antibiotics, notably aminoglycozides that are important antibiotics used intravenously to treat gram negative bacteria, diuretics, and several cancer drugs may lead to CKD [4].

Birth defects and anomalies of the kidneys such as polycystic kidney disease or deformation of the kidneys are rare causes of CKD. Hereditary renal and metabolic conditions and family history of chronic kidney disease are also risk factors. Smoking is regarded as an independent risk factor of CKD [2, 4].

Certain toxic chemicals (other than prescribed drugs) include heavy metals such as cadmium, mercury and platinum may lead to CKD [8]. Other chemicals include illicit drugs, toxic herbs, polluted water and food additives. Diseases or damages to renal veins or arteries include rare thrombo-embolic diseases with or without infarction and bleeding are rare but important causes of kidneys damages [4, 8]. .

Malignancies may cause kidney damages either directly by dissemination and metastasis or directly by pressure, infection (weakening of the immune system), blood flow disturbance (the tumor may invade the renal arteries or the renal veins), bleeding, cellular lysis, hematological manifestation, the production of immune globulins and amyloidosis. Multiple myeloma is among the most common cancer-related causes of renal disease (both acute and chronic) by several mechanisms including cell lysis, drug toxicity, auto-antibodies and amyloidosis. Amyloidosis may be the cause of the death of the person. Systematic amyloidosis and para-proteinuria are associated with chronic inflammation with or without malignancies and can cause either acute or CKD [2, 4].

Trace mineral accumulations (notably copper) in the kidneys are possible (Wilson's disease). The patient will also suffer from liver and cardiac failures, which complicate CKD [8].

Other causes are often manifested with acute renal failure, but may progress into CKD if repeated or caused irreversible renal damages. These causes include multisystem organ failure after shock including burns, septic shock and sepsis and other causes such as severe bleeding (repeated, if the person survives), acute pancreatitis, repeated cell lysis and uncontrolled gout, hemolysis and rhabdomyolysis (muscle lyses), snake poisoning and any poison that lead to disseminated intravascular coagulation and eclampsia (associated with pregnancy) [2, 4].

Pathophysiology of CKD

The healthy rate of renal blood flow is about 400 ml/100g of tissue per minute and is greater than other well-supplied organs such as the liver, the heart, the lungs and the brain [1]. This explains why the kidneys are very vulnerable to harmful substances or antibodies.

Auto immune diseases are often manifested in the kidneys because of their vulnerability to antibodies [1, 7]. An auto immune disease or a para-neoplastic syndrome (caused by abnormal antibodies made by cancer cells as in the case of blood cancers and myeloma) may affect the kidneys much faster, or may manifest solely in CKD, because the sensitivities of auto antibodies and their damaging effects are higher than those of glycation [9]. An immunological reaction with or without complex antibodies is the most potent cause of kidney damage [10], followed by tissue hypoxia and ischemia, exogenic agents (drugs), glycation (poorly controlled diabetes mellitus), paraproteins (abnormal proteins and immunoglobulins as in case blood cancers like multiple myeloma), and finally genetic defects.

Based on statistical data, diabetes and hypertension are the two most common causes of CKD in the USA [6, 11] and diabetes mellitus remains the number one worldwide [9]. Uncontrolled Diabetics is likely to cause CKD by a combination of mechanisms. Factors that increase the risk of CKD in diabetics include glycation which directly affect the kidneys. Other co-morbidities include increased risk of cardiovascular disease such as hypertension, recurrent infections, poor nutrition, and other metabolic abnormalities including gout and acid-base imbalances [3-5, 9].

Glomerular filtration requires a high pressure of blood flow at normal conditions which makes the nephrons very sensitive to hemodynamic injuries. This would explain the damages caused by chronic hypertension or chronic plasma overload to the kidneys.

The descending passage of glomerular filtration and the pressure required makes the tissues very vulnerable to extreme blood flows and volumes. If the blood flow is in excess or if the arterioles are dilates, this can damage the nephron directly or via rupture and bleedings. If the blood flow is reduced, this can damage the nephrons by causing ischemic disease and infarction. This mechanism is also seen in patients with cardiovascular diseases, chronic hypertension and other lesions of the renal blood flow. Glomerular filtration membranes have negatively charged molecules. This acts as an electrostatic barrier and regulator to major plasma electrolytes. A disruption in plasma electrolytes may also damage such a barrier [1, 10].

Glomerular sclerosis and fibrosis of the tubules are seen in most cases of advanced CKD. Activation of inflammatory cells and involvement of cytokines lead to fibrosis and damage to the renal tissue and the kidneys become unable to eliminate waste properly [10]. Wastes are toxic (including nitrogen waste and many drugs). The retention of nitrogen waste leads to uremia.

Uremia and dialysis-induced overload of chemicals lead to an increase in oxidative stress and inflammation via the retention of inflammatory cytokines in the plasma. Local inflammation and cellular infiltration are associated with and complicated by systematic inflammation and oxidation which complicates CKD [12- 14]

Based on the physiology of the kidneys, CKD also leads to disruption in water, sodium and potassium homeostasis. CKD also leads to disruption of the renin-angiotensin-aldosterone (RAA) and the vasopressin or anti diuretic hormone (ADH) systems. This worsens sodium, potassium and blood pressure disturbance. CKD is also associated with calcium and phosphorus imbalance (vitamin D is activated in the kidneys), hyperparathyroidism and bone dystrophy which further complicate kidney failure; acid-base balance disturbance (mild acidosis). Microcytic anemia with low hemoglobin and hematocrit is caused by the loss of erythropoietin production is

seen in CKD. CVD either as causes or consequences of CKD (such as hypertension) are exacerbated. Malnutrition (which partially explains why individuals with low BMI and low albumin and LDL have higher risk) and systematic abnormalities (neuropathies, gastrointestinal disorders, CVD, skin and other generalized and metabolic abnormalities) are often seen in CKD [2, 4, 15].

Effects of CKD on lipid and glucose metabolism due to oxidative stress and inflammation are common. Insulin resistance (IR) is also found among non-diabetics with CKD and ESRD. The toxic uremic state and the co-existing anomalies including obesity, dyslipidemia, metabolic acidosis and vitamin D deficiency contributes to insulin resistance and complicates the treatment of end stage renal disease [16].

Inflammation, Oxidative Stress and CKD

It is established that inflammation and oxidative stress are pathologies among all causes of CKD [13]. They are also linked to IR and dyslipidemia [13, 14].

An old hypothesis flourished in the 1980s and proposed that ESRD causes an increase in immune cytokine interleukin-1 (Il-1) production as a result of exposure to celluloic membranes and that leads to hypotension in patients with hemodialysis, an increased risk of cardiovascular disease, and protein-energy wasting [14]. Correro and Stenvinkel stated several mechanisms are involved in inflammation among patients with ESRD. Such mechanisms include elevated C-reactive protein production (CRP) in uremic patients, infectious agents, periodontal inflammation, and retention of inflammatory cytokines, advanced glycation (notably in diabetics) and oxidative stress (pro-oxidants) [14].

The inflammation-weight loss- hypotension may also explain a paradox that ESRD patients with higher BMI or higher blood pressure have better prognosis. Weight and blood pressure may reflect nutritional and cardiovascular status because weight loss and low blood pressure indicate poorer prognosis among patients with ESRD [17, 18]. Inflammation also leads to destruction of vascular walls, increase in coagulability and abnormal plasma lipid homeostasis [10, 19, 20].

IR adds as a novel risk factor for inflammation as stated [16] and is modulated by overproduction of inflammatory cytokines [21]. IR is seen among obese with metabolic syndrome, but is also associated with wasting, weight loss and dyslipidemia among patients with ESRD [21]. A cohort study by H.-T. Kang et al. in 2010 covered 8411 participants from rural Korean background found that IR was associated with higher TG /HDL ratio among both genders independently of waist circumference [22].

Oxidative stress is strongly linked to inflammation, abnormal lipid profile, atherosclerosis, adiposity and hypertension in patients on hemodialysis [13, 23]. The toxic uremic state, the chemicals in dialytes and the lacking of antioxidant consumption such as vitamin E may worsen oxidative stress in uremic patients [13]. Lipoperoxidation and inflammation may also be associated with adiposity in severe CKD [23].

The nutritional status of patients with CKD and ESRD may be inversely related to the risk of oxidative stress and should be accounted for. Some overweight patients expressed a better survival rate which was suggested as a result of adequate calories and nutritional intake that would include anti-oxidants [16, 17].

Effect of CKD and ESRD on Lipid Profile

Dyslipoproteinemia and oxidation contribute to modification of LDL to oxLDL. Increased LDL and triglyceride-rich lipoproteins are atherogenic. The accumulations of LDLs and their entry inside the arterial wall leads to further modification such oxidation or aggregation. LDL oxidation and aggregation play a pivotal role in atherosclerosis [19, 24]. Modified LDLs trigger inflammation via stimulation of immune cells and activating adhesion molecule expression of monocyte chemotactic protein -1 (MCP-1). MCP-1 triggers the transformation of monocytes into macrophages. Macrophages act as scavenger cells engulfing oxidized LDL, and are converted into foam cells [25]. Foam cells and macrophages produce growth factors and cytokines including interleukin -6 (IL-6), tumor necrosis factor (TNF), and several adhesion molecules [26]. The adhesion molecules bind to monocytes and recruit new cells creating a vicious cycle of inflammatory process [26]. This leads to narrowing of the arteries and increases the risk of their rupture, dissection or occlusion.

This cycle may be countered with an adequate level of HDL notably HDL3-C [12]. HDL is recognized as an antioxidant and anti-thrombotic that inhibits LDL-oxidation and monocyte chemotactic protein -1 or MCP-1 production. A functional HDL may reverse the process of atherosclerosis because HDL removes cholesterol from cells of arterial walls [27, 28] (Figure 1.1)

Patients with ESRD are at higher risk of atherosclerosis than the normal population. CVD exists as co-morbidity, either as a cause (such as hypertension and metabolic syndrome) or as a result of the conditions of patients of CKD. A cardiorenal syndrome has been well-described by the American Heart Association [29]. Progressive increase in inflammatory markers and inflammation also leads to destruction of vascular walls, as well as an increase in coagulability, chemotaxis and abnormal plasma lipid homeostasis, which creates a vicious cycle [19, 20].

A study was conducted comparing 55 patients in stage 5 CKD. Thirty one patients were on hemodialysis (HD) and 24 patients were on peritoneal dialysis (PD). The study found that patients with ESRD tend to have low HDL –cholesterol and elevated levels of VLDL and IDL which cause an increase in triglyceride levels. The study also revealed that hemodialysis (HD) patients tend to have less elevation of cholesterol and apo-protein B than PD patients, but the cardiovascular outcome was similar in both groups. Patients with CKD also tend have 10-fold elevation of oxidized LDL (oxLDL) [12].

A proposed mechanism for such abnormalities was the activation of free fatty acids (FFAs) delivery to the hepatocyte as well as their oxidation and esterification to cytosolic triglycerides or VLDL. This leads to an increase in apo-B production. The increase of apo-B production leads to an increase in atherogenic VLDL and IDL. This increase in free delivery and activation of FFAs is attributed to exposure to dialysate, IR, and/or increase in glycation (exposure to glucose) [30] as well as generalized oxidative stress and inflammation [12]. Several studies cited the importance of IR in pathology of CKD and dyslipidemia even in non-obese and non-diabetic patients [12, 16, 22, 30]. Studies also found an association between a higher level of oxLDL and a decrease in the activities of the enzymes with HDL-related antioxidant effects. There was a decrease in HDL3-C/HDL2-C ratio in both HD and PD. The effects of these abnormalities in lipid profile, and increase in apo-B lipoprotein and oxLDL lead to an increase in oxidative stress and atherogenesis.

Alteration of Major Lipoproteins in Patients with CKD and ESRD

CKD and ESRD affect individual lipoproteins and their metabolism. I list some of them and shall discuss pertinent lipid metabolism in greater detail in the discussion section.

Alterations in metabolism of triglyceride-rich lipoprotein in predialysis and dialysis patients are common. VLDL, chylomicrones and their remnants increase beginning in early stage CRD. Serum triglycerides tend to be higher after meals among patients with CKD than those who are healthy. This is often attributed to IR of patients with CKD [31]. Reduction of catabolic rate of VLDL is also associated with low lipoprotein lipase activity and down regulation of the enzyme gene [32, 33]. A decrease of apoprotein C-II/C-III leads to more inhibition of lipoprotein lipase LPL (Apo C-III inhibits LL while Apo C-II activates it) [33]. Secondary hyperparathyroidism and increased liver production of TG may also be involved [34]. The use of low molecular heparins in HD patients contributes to abnormal catabolism of triglyceride-rich proteins, while the use of high flux polysulfone or cellulose triacetate reduces triglycerides in HD patients [35].

Alterations in LDL metabolism in predialysis and dialysis patients are common. They are influenced by heavy proteinuria. The loss of albumin affects the gene expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), cholesterol 7 alpha hydroxylase, and cholesterol receptors in the liver [36]. Acquired LDL receptors deficiency may also be a factor in nephritic proteinuria [36]. Acquired CKD (in absence of proteinuria and glomerulostenosis) does not alter hepatic LDL receptor gene expression, but may alter LDL metabolism [32]. The loss of albumin in the urine may also stimulate the liver to produce lipoprotein B and other LDL-related proteins. This occurs more often in peritoneal dialysis patients. A microalbunirunia is also associated with higher risk of CVD [32, 37]. Up regulation of Acyl-CoA cholesterol

acyltransferase or ACAT (the enzyme that esterifies free cholesterol in hepatocytes) has also been seen in nephrotic syndrome patients along with increase in HMG CoA reductase and down regulation of cholesterol 7-alpha hydroxylase (the rate-limiting enzyme in bile acids synthesis) [32, 38].

HDL is also altered in predialysis and dialysis patients [32]. HDL-C plasma level is reduced in patients with CKD and ESRD. Mechanisms of HDL alteration involve decreased levels of apoprotein AI and AII, reduced activity of Lecithin—cholesterol acyltransferase or LCAT (the enzyme that esterifies of free cholesterol in HDL), and an increase in Cholesteryl ester transfer protein (CETP) activity [32, 39]. As stated before, oxidative stress and inflammation are often increased in patients with CKD and ESRD and contribute to dyslipidemia which further affects HDL antioxidation activity [20, 21, 23].

Treatment Options of ESRD

Treatment of CKD includes treating the individual abnormalities, symptoms and co-morbidities that caused CKD and/or are associated with and caused directly by CKD. The treatment provides a reliable method to remove excess wastes, subside for the roles of the kidneys by dialysis or kidney transplant. It also involves treating co-morbidities that caused inflammation, contributed to CKD or worsened the complications of CKD such as diabetes, hypertension, malnutrition, infections, and other chronic diseases. Educating patients and providing them with support and care are of importance because the disease is chronic and may have severe psychological and social consequences. PD and HD should be assessed for safety and sterility of chemicals, lines and dialysate (risk of infection), volume of dialysis and dialysate and for thrombo-embolic

complication of the graft or the venous fistula. [40]. The use of medicinal and nutraceutical agents in CKD may also be warranted.

Medical options may vary and include the use of lipid-lowering drugs to treat hypercholesterolemia patients and improve overall cardiac mortality rate among this group of patients [40, 41]. It also involves the use of anti-hypertensive medications such as ACE inhibitors; the use of vitamin D to replace inadequate activation of vitamin D; the use of erythropoietin injection to treat anemia; to eliminate and treat other causes of anemia which may implicate the cardiovascular system and the renal system; the use of anticoagulation (baby aspirin); to monitor the coagulability status of patients; and to use selective anti-inflammatory medications that act on some interleukins and TNF [40].

Nutritional options are also considered. Vitamin D (stated before) is used. The use of omega 3 fatty acids that are derived from fish oil (EPA and DHA) may be warranted because of their anti-inflammatory status. Two studies [42, 43] found improvement in TG level and lipid profile when using omega-3 fatty acids. Omega-3 fatty acids also improved C-reactive protein level [42].

The effects of vitamin E supplements in CKD and ESRD were not consistent. An animal study found an attenuation of diabetic nephropathies by tocotrienol isomers in diabetes-induced rats [44]. An Italian clinical study found a decrease in LDL oxidation in patients undergoing HD. Vitamin E (tocopherol) supplementation improved LDL resistance to oxidation but it did not modify lipid profile [45]. Another human study involving vitamin E (tocopherol) supplements did not show an improvement in C-reactive protein and pregnancy-associated plasma protein –A (PAPP-A) markers [46]. A study using tocotrienol rich fraction (TRF) supplementation showed

that TRF increased expression of apo A1 and reduced expression of inflammatory marker C-reactive protein [47].

The use of soy supplements such as isoflavone, vitamin C, α-tocopherol, selenium, α-lipoic acid, coenzyme Q 10, red grape juice, genistine and other anti oxidants and phyochemicals have been tried [48]. Lists of antioxidants and validations of such studies may be found in the Cochrane collaboration review [49].

The use of HD, PD or kidney transplant are the lasting choices to treat ESRD (HD is a lot more prevalent than PD in the West). Kidney transplant remains an option considering the patients co-morbidities, immune status, and age.

Vitamin E

Vitamin E isomers are fat-soluble compounds that can be stored. They are classified as vitamins because humans cannot synthesize them and because they are essential [50]. The first form of vitamin E was first isolated by Herbert Evans and Katherine Bishop at the University of California from dark leafy vegetables and the vitamin was known to support fertility. The name tocopherol was derived from the Greek *tokos* ("childbirth") and *phero* ("to give forth") and *ol* for its alcohol properties [51].

Vitamin E was found to exist in many isomers. Tocotrienol isomers (T3) were found in seed endoplasm of monocots such as wheat, rice and barley. Unlike tocopherol isomers which are found in many plants, tree nuts and dark greens, T3 are almost exclusively found in seed endoplasm. Palm oil and annatto contain substantial amounts of T3 [52].

The *trienol* or *trienes* relates the three unsaturated double bonds and the side chains that differ T3 from tocopherol isomers. There exist a total of four tocopherol isomers and four tocotrienol

isomers and are known collectively as tocochromanols that are regarded among the most effective of lipophilic phenolic antioxidants [50, 53].

All eight vitamin E isomers contain a chromanol ring. Its hydroxyl ring is responsible for donating hydrogen that reduces free radicals. The hydrophobic side chain allows their entry into cell membranes. Tocochromanols occurs in alpha, beta, gamma and delta forms or α, β, γ and δ. We have α, β, γ and δ tocopherols and α, β, γ and δ tocotrienols [53] (Figure 1.2).

The biosynthesis of vitamin E occurs mainly but not exclusively in photosynthetic organisms because certain bacteria such as *Escherichia coli* are capable of synthesis of tocochromanols. Vitamin E was classified as an antioxidant and was labeled "factor 2" by Schwarz in 1965 [54].

The abundance of α tocopherol and its anti-oxidant property led scientists to ignore non-tocopherol forms of vitamin E. T3 have been poorly studied and vitamin E has been incorrectly associated with tocopherol [51, 55].

Mechanism of Action of Vitamin E Isomers

The major anti-oxidant function of vitamin E is scavenging lipid peroxide radicals and protecting fatty acids in cell membranes from peroxidation including light-induced lipid peroxidation during germination and photosynthesis [56]. The Chromanol ring is a hydroxylated aromatic ring with 15 carbon-tails which characterizes this group of vitamins. It is the active radical quenching part [57].

Vitamin E isomers are potent antioxidants. Polyunsaturated fatty acids (PUFAs) in cell membranes are more prone to light oxidation or chemical oxidation. Lipid peroxyl radicals react with lipids to produce hydro peroxides and oxylipins which may alter gene expression. The steps

of this reaction are prevented by vitamin E and carotinoids. The fact that vitamin E is hydrophobic permits it to act on lipids in cell membranes and that leads to a better cellular stability, and membrane stability is recognized as a function of vitamin E [56, 58].

Free radicals that are generated by one-electron transfer processes may also be produced by drugs, toxic chemicals, heavy metals, and ionizing radiation [58]. Their production may damage lipids in cell membranes and other particles, cellular proteins, carbohydrates and DNA. There is a relation between those chemical reactions because oxidized lipids would alter gene expression and thus protein synthesis. Oxidized vitamin E that picks up the free radicals is reduced back to its original form with the presence of ascorbic acid (vitamin C) and glutathione [56]. This is another important point to conclude that vitamin E acts in concert and harmony with fat-soluble vitamin A and other carotinoids and does require, in return, water soluble antioxidant vitamin C. A nutritionally-sound diet should include a harmony of the three anti-oxidant vitamins (Figure 1.3).

Vitamin E isomers in animal cells exert similar anti-oxidative cycles as observed in plants. They also aid in stabilizing animal cell membranes. The redox cycle involves other compounds notably coenzyme Q 10 because there is a direct anti oxidative effect of vitamin E in mitochondria as well. It has been shown that coenzyme Q 10 acts as peroxyl scavenger that is formatted by superoxide-driven reaction. This prevents the reaction of pro-oxidant of phenoxyl radicals of vitamin E in LDL [59]. Actions of T3 have also been attributed to their unsaturated isoprenoid tails that lead to a better uniformity of the distribution in membrane layers, stronger effectiveness on their lipids, effectiveness on radicals and better recycling activity and inhibition of lipid oxidation [60].

T3 penetrate cell membranes more efficiently than tocopherol isomers and their unsaturated chains make them more accessible to tissues that are rich in saturated fatty layers including the brain and the heart [61]. It was found that T3 in particular have significant reduction of thiobarbituric-acid-reactive substances (a product of oxidized PUFA) in the serum of patients with carotid stenosis [62].

Absorbance and Availability of Tocopherols and T3:

When vitamin E is obtained from food, it will be transported to the liver to be sorted out to its isomers [58]. The endogenous lipoprotein that carries most of vitamin E inside the body is LDL and triglyceride-rich fractions (TGRF) [63, 64].

After normocholesterolemic women received an oral vitamin E capsule (containing 77 mg α-tocotrienol, 96 mg δ-tocotrienol, 3 mg γ-tocotrienol, 62 mg α-tocopherol and 96 mg γ-tocopherol), the isomers level peaked four hours after. It was found that α-tocopherol had the largest concentration among all six isomer, α-tocotrienol had the largest concentration among T3 and that T3 were found in all lipoprotein notably in LDL [14]. It was concluded that α-tocotrienol is the most available tocotrienol isomer in healthy human plasma lipoproteins while α-tocopherol was the most abundant of all [63, 65].

Once released from lipoproteins to target cells, they will bind proteins that aid in transporting vitamin E isomers inside the cytosol. When inside the cell, vitamin E is also placed in the hydrophobic site of cell membranes.

The multifaceted roles of T3

Current studies reveal that the vitamin E family isomers have many functions in addition to their anti-oxidative functions, such as an effect on platelet adhesion and kinase enzymes [51].

T3 have structural activities unseen in those of tocopherols. The double-bond carbons at 3, 7, and 11 that are found in T3 allow them to have better and wider activities than tocopherol isomers because they can better penetrate the cell membranes. The three double-bonds and unsaturated side chains allow them easy access and movement to cell membranes [60, 65]. These characteristics are related not only to the saturation of the chain, but also to the methylation of the ring [65]. It was found that δ-tocotrienol has a higher or equal biological action to those of γ-tocotrienol; which in return are more biologically active in descending order than those of α-tocotrienol, δ-tocopherol, and α-tocopherol [66].

Molecular targets of T3 are either modified by direct binding, by modulation or by indirect modulation [53, 67]. Modulation includes transcriptional, translational, post-translational level or may occur via direct interactions with various cellular targets. Examples of direct binding include the effect on HMG-CoA reductase.

Inflammatory transcription factors and their genes are modulated indirectly. Studies demonstrated that T3 possess anti-oxidant, anti-inflammatory, antiproliferative, antisurvival, antiangiogenic, and pro-apoptotic activities [51, 53, 67]. This makes T3 more pleiotropic (having many multifaceted physiological effects) nutrients than tocopherol isomers.

The antioxidant activity of T3 includes the induction of various enzymes such as superoxide dismutase and glutathione peroxidase. Animal studies found that T3 notably γ-tocotrienol expressed antioxidant activities as well as antihypertensive activities [53, 68].

Animal studies also demonstrated that T3, notably γ and δ, carried more wide anti-proliferative and anti-cancer activities on breast cancer cell lines than α-tocopherol [69]. Studies also found that T3 carry anti inflammatory activities including that of the effect on activation and transcription factor NF-κB, the suppression of the expression of TNF, interleukins 1, 6 and 8, induction of nitric oxide synthase and cyclo oxygenase 2 (COX 2). The effect of gamma-tocotrienol on NF-κB that transcribes for many inflammatory markers is also cross-linked to its anti-cancer anti-apoptotic effect [70].

There is a significant importance of the anti-oxidative and the anti-inflammatory properties of T3. Patients with CKD and ESRD have increased inflammation and oxidative stress. These changes are fundamental parts of the pathology and pathophysiology of an abnormal lipid metabolism in these patients [71].

This section will review findings in literatures that are pertinent to the effect of T3 and lipid metabolism and lipoproteins. As noted earlier, T3 suppress HMG-Coenzyme-A reductase, the rate limiting enzyme in cholesterol synthesis. The hypocholesteromic effects of T3 were attributed to the post-transcriptional modification of the rate limiting enzyme HMG Coenzyme A reductase. Mechanisms involve enhancing the degradation of the enzyme and reducing the translation of its mRNA. There have also been links between anti-tumor property and HMG-CoA reductase inhibition of tocotrienol [53].

Lipid lowering effects of T3 were first studied on animals and cell lines. Two studies by Qureshi (1991, 2001) [72, 73] demonstrated that T3 in rice bran and tocotrienol-rich-fraction (TRF) of palm oil may lower plasma cholesterol in pigs with hypercholesterolemia. The pigs were fed a standard diet with 50 micrograms/g TRF obtained from palm oil. There was a 60% reduction in LDL-cholesterol, 44% reduction in total cholesterol with 26% reduction in

apoprotein B. This effect lasted in hypercholesterolemic pigs after 8-week consumption of the control diet. Animal studies also demonstrated that T3 suppress HMG-CoA reductase with better protective effect of gamma and delta tocotrienols than alpha and beta isomers. Qureschi also demonstrated that T3 inhibits lipopolysaccharide-induced cytokines in macrophages in female mice and that T3 were superior in their enzymatic activity than that of α-tocopherol in female mice [74].

Another study by Khan et al on Syrian hamsters in which tocomin (mixed of 4 isomers of T3) was administered to the animals 10 days before and 12 hours after bacterial lipopolysaccharides or 24 hours after zymosan or turpentine to induce inflammatory reaction. Results showed that T3 reduced plasma lipoprotein lipids, cholesterol, apoprotein B, small dense LDL and LDL in animals who were hyperlipidemic [75]. Not all early T3 studies showed lipid lowering effects of T3. Mensink et al conducted a double-blind placebo-controlled parallel trial on 20 men with elevated plasma cholesterol levels. The study showed that T3 had no effect on plasma lipoprotein status [76].

T3 cell studies also demonstrated direct effects on triglyceride. Burdeos et al found that T3 attenuate triglyceride accumulation in HepG2 and rats, and that γ-tocotrienol down regulated fatty acid synthase in Hepatoma G2 cells that received it. There was similar expression in the protein expression of the gene that codes for that enzyme [77]. Zaiden et al showed that gamma and delta T3 reduce hepatic triglyceride synthesis and VLDL secretions in human hepatocarcinoma cell lines. They suggested that discrepancies between in vivo and in vitro studies may be due to the rates of post-absorption of T3 [78].

An animal study by Kuhad et al found that T3 were more effective than α-tocopherol in diabetic rats with nephropathy (damage in the kidneys). T3 were given at a dose of 25, 50 and 100

mg/day to streptozotoci-given diabetes-induced rats. This prevented the progress of diabetic-nephropathy in a dose-dependent fashion. Adding insulin to T3 produced better effects than insulin alone in treating diabetes [44].

Clinical studies were thus warranted to demonstrate the effects of T3 on human lipid profile. It has already been stated that early studies of T3 on humans were not only limited, but also gave contradictory results [76, 79]. Qureshi conducted a double-blind crossover 8-week human study. The subjects received either a 300 mg/corn oil or a 200 mg TRF capsule per day. The results showed beneficial effect of TRF and a 31% reduction of plasma cholesterol was noted in seven hypercholesterolemia subjects during the four-week of receiving the TRF supplementation [80]. Chen and Qureshi also demonstrated that isolated T3 from rice bran had a cholesterol-reducing effect. Such an effect also has been noted in amaranth oil. The study of which hypercholesterolemic individuals placed on the American Heart Association step-1 diet and also receiving statin drug lovastatin and TRF resulted in a significant improvement in lipid parameters with an increase in HDL/LDL ratio by 46%, and without side effect of the statin. The study showed that a dose of 100 mg/day of TRF lowered serum total cholesterol, LDL-cholesterol, triglycerides and apoprotein B, suggesting the possible use of a smaller dose of TRF plus American Heart Association step-1 (AHA step-1) diet to control risk factors of coronary heart disease in hypercholesterolemics [81].

Unlike the success studies of Qureshi, there existed studies demonstrated that T3 did not successfully affect cholesterol homeostasis [76, 79]. After Mensink's study 1999, another study by Mustad et al, (2002) demonstrated that supplementation with 3 different tocotrienol supplements did not improve lipid levels and CVD risk factors in humans with hypercholesterolemia [79].

Equally interesting was a tocotrienol study on HDL-cholesterol. TRF supplementation in healthy older adults raised plasma HDL as early as 3 months [82]. The study involved recruiting 62 subjects from two different age groups: 35-49 years and above 50 years of age. Each group was given either TRF or a placebo at random for six months. The result showed a statistically significant elevation in HDL-cholesterol after 6 months of TRF supplementation as compared to the placebo among the younger group and an improvement of HDL-C to total cholesterol ratio in both groups. It also showed reduction in oxidative stress.

Another study demonstrated that supplementation with TRF altered plasma levels of apoprotein-A1 precursor (apo A-1 unique to HDL), apolipoprotein E precursor, and C-reactive protein (CRP) precursors in young (32 ±2 year old) and old (52±2 year old) individuals. The study concluded that TRF does not only alter and increase plasma levels of tocopherol and T3, but also up-regulated apoA1 and down regulated CRP in the older individuals. Such results are favorable for atherosclerosis prevention [47]. The kidneys are the major organs that filter apo-A1. ApoA1 proteins are released by the action of lipase on HDL, which are enhanced by CETP and are catabolized in the kidneys [83].

T3 and Lipoprotein Metabolism

The previous section included studies that covered cell lines, animals and some clinical studies. It is of an importance to state major findings obtained from literatures on the effects of T3 on lipoprotein metabolism because dyslipidemia and CVD are higher in patients with CKD and HD [84].

Lipid lowering effects of T3 were observed in animal studies, and revealed that T3 suppress or down regulate HMG-CoA reductase, lower synthesis and increase degradation via

post transcriptional effect [85]. Human studies demonstrated stronger effect of gamma and delta tocotrienol. Effects of T3 on HMG-CoA reductase were contradicted by high dose of alpha tocopherol. T3 also lowered apoprotein-B levels [84, 86]. As previously stated, data from T3 studies were not always consistent [76, 79].

The contradictory effects of T3 on plasma lipids may be attributed to the followings: 1) Differences in absorption because absorption may increase when taken with meals. 2) Dose-related effect (in many cases. lower doses may have been required). 3) Clinical condition of the individuals. 4) Difference of tocotrienol isomers absorption between animals and humans. 5) Duration of the study (not long enough to produce results). 6) Metabolism of T3 and their effect may be varied [84].

CRP, an acute phase protein and a major inflammatory marker in CVD and is implicated in re-stenosis of coronary arteries. CRP is synthesized in the liver and regulated by tumor necrosis factor-α TNF and IL-6 [87]. Tocotrienol isomer gamma showed 20-50 % higher potency in reducing CRP than that of tocopherol alpha in diabetics with CVD [71, 87]. T3 also inhibited LPS-induced secretion of TNF and IL6 in macrophage of mice and a low concentration of alpha tocotrienol reduced IL-6 and cytokines by TRF alpha, gamma, and delta tocotrienol [74]. Palm oil TRF also reduced the transcription of IL-4, IL-8, TNF-α and NFκB [88].

T3 alpha showed antioxidant activities such as an effect on peroxyl radical scavenging activity being 1.5 times higher than alpha tocopherol, effect on Fe (II)-NADPH-induced lipid peroxidantion of alpha T3 is 40 times higher than that of alpha-Tocopherol [60, 84]; and effect on cytochrome P450 is 6.5 times higher than tocopherol [89].

T3 have antihypertensive and anti-diabetic effects in rats [53, 84]. Both diseases are major risk factors for CVD and CKD. Glycation that is associated with poorly controlled diabetes may

be improved or reduced by T3. Gamma and delta T3 had demonstrated a strong effect in advanced glycosylation-end products reduction in non-diabetic rats [90]. Vitamin E (including α-tocopherol) also reduces the serum level of age glycation products (AGE) in patients on HD [91].

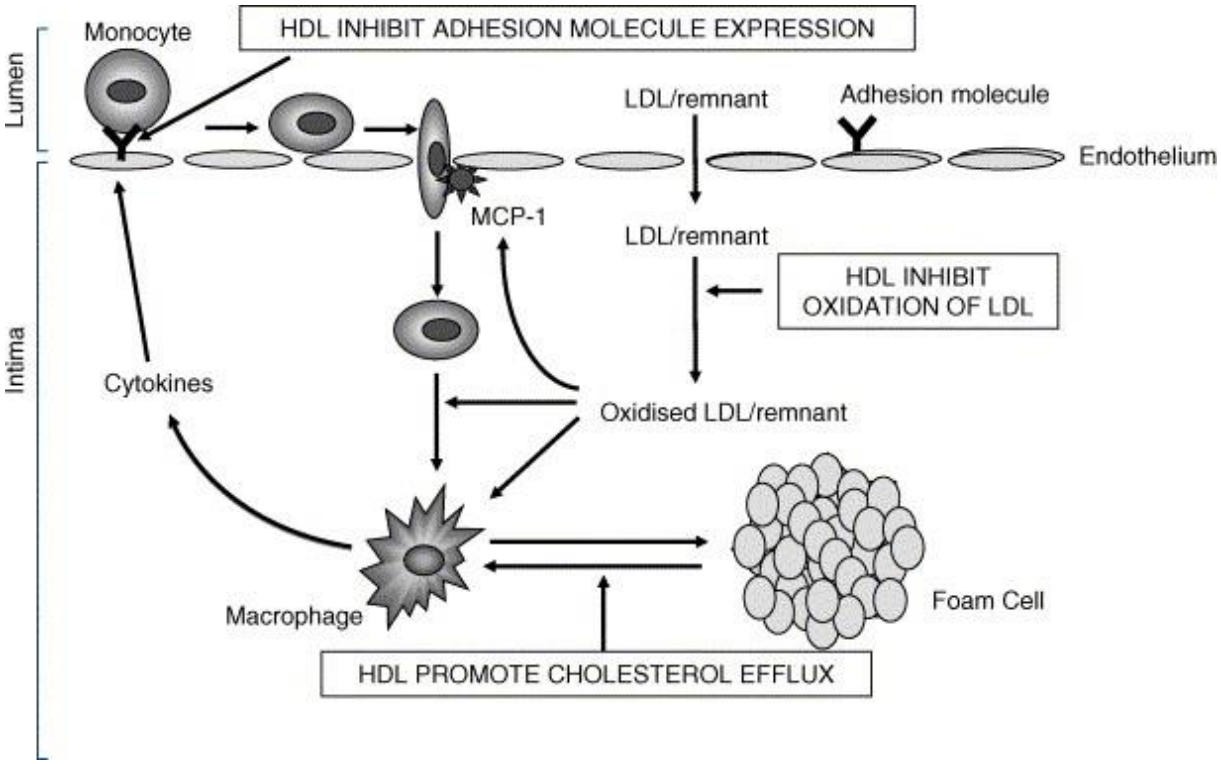
The antihypertensive activity of T3 may be attributed to its anti-oxidant effect and its effect on nitric oxide [68, 92]. This anti-oxidant effect plays a role in both diabetes and hypertension. T3 have an effect on adhesion activity. Cellular adhesion is affected by oxidative stress and is an essential part of the mechanism of atherosclerosis and CVD that shall be discussed later. Alpha-tocotrienol is more effective than alpha-tocopherol as a result of the suppression of selectin and vascular cell adhesion molecule that are responsible for foam cell formations. T3 are collectively more effective than alpha-tocopherol [93].

Potential Clinical Uses of T3

Potential uses of T3 as a preventive drug have been reviewed with consideration to their structure and their bioavailability. Literatures listed some potential cardio-protective implications of the uses of T3 [84, 86, 94-96]. The use of T3 on atherosclerosis in patients with carotid stenosis with palm oil revealed that 30% of patients showed regression 6% showed progression and 56% showed no change. Direct suppression of atherosclerosis was seen in animal studies. These effects have been attributed by various mechanisms (effect on lipids, HDL, cytokines, antioxidant, anti-inflammatory, anti-chemo taxis, anti-adhesion and anti-glycosytion) [84, 86, 94, 96]. Tocopherol in experimental animals did not regress atherosclerosis. The conversion of tocopherol to a pro-oxidant that may also lower vitamin C reduces the effect of glutathione peroxidase enzyme.

T3 also affect plaque stabilization, as a result of their lipid-lowering effect, anti-inflammatory and anti-adhesive. Despite the limitations of human clinical studies, animal studies

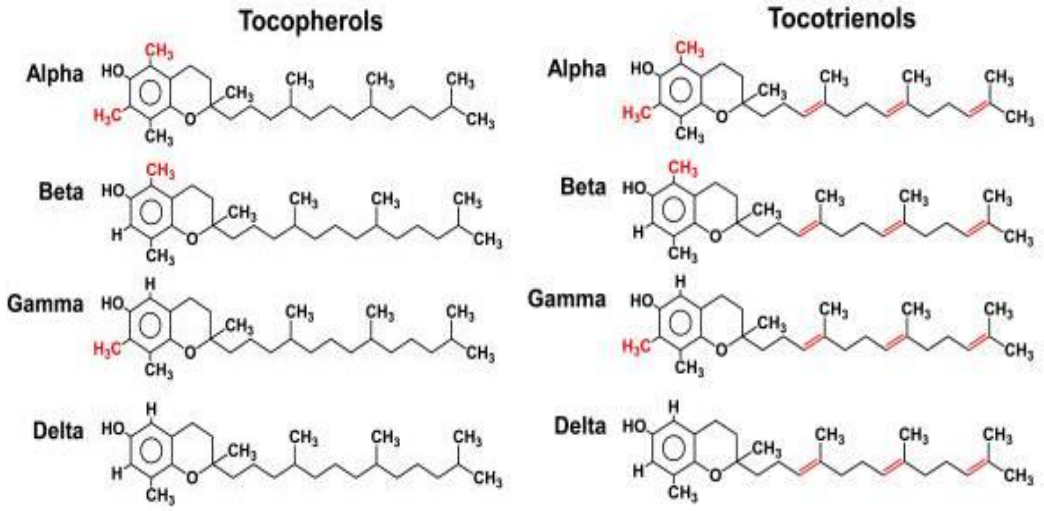
were promising. The effect on expression of MMPs and cell adhesion may be promising. There was an ischemia-reperfusion effect because free radicals have negative effect on post-ischemic disease. T3 might affect ischemic reperfusion due to its anti-oxidant effect. Anti-thromboembolic effect of T3 (effect on platelet aggregation), and effect of post angioplasty and post-cardiac injury (anti-proteasomes) were also studied [86, 94]. Not all T3 have equal effects. It was found that α and γ T3 have better cardio protective effect than the other two isomers. The lipid modulating effect of T3 was notably seen in the γ and δ isomers [84, 86].



Source: Barter, 2005

Figure 1.1: HDL and atherosclerosis

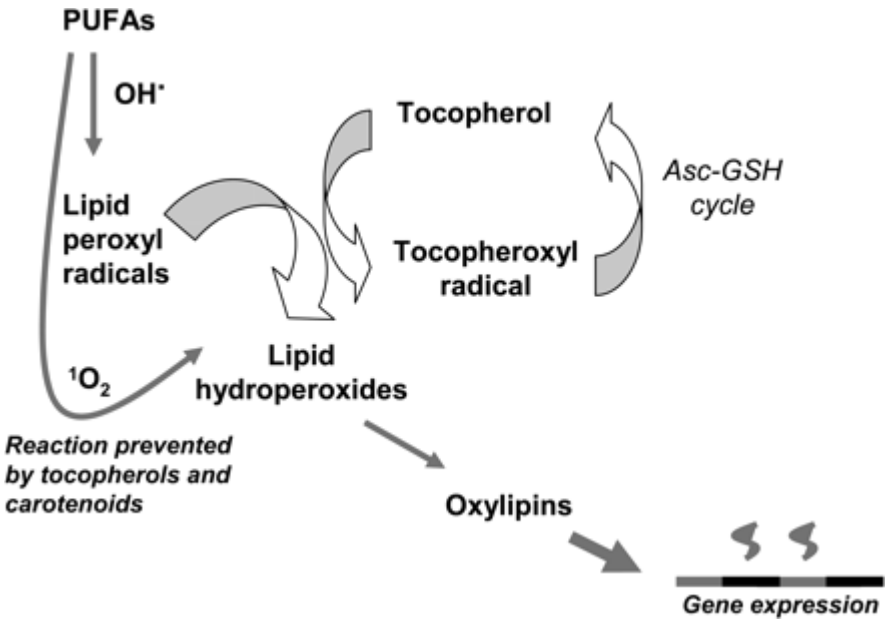
Inhibition of atherosclerosis by high density lipoprotein HDL removes cholesterol from foam cells, inhibits the oxidation of low density lipoprotein, and inhibits adhension molecule MCP-1 and monocyte chemotaxis protein.



Source: Aggrawal et al, 2010.

Figure 1.2: Structures of tocopherol and tocotrienol isomers.

All eight vitamin E isomers contain chromanol ring. Its hydroxyl ring is responsible for donating hydrogen that reduces free radicals. The hydrophobic side chain allows their entry into cell membranes. Tochochromanols occurs in alpha, beta, gamma and delta forms or α, β, γ and δ. We have α, β, γ and δ Tochopherols and α, β, γ and δ Tochotrienol isomers. The double bond carbons at 3, 7 and 11 that are found in tocotrienol isomers allow them to have better and wider activities because they can better penetrate the cell membranes. Actions of tocotrienol isomers (T3s) have been suggested due to their uniformity of the distribution in membrane layers, stronger effectiveness on their lipids, and effectiveness on radicals, better recycling activity and inhibition of lipid oxidation.



Source: Falk et al, 2010.

Figure 1.3: The antioxidant mechanism of vitamin E.

Vitamin E (Tocopherol) isomers regulate redox homeostasis and gene expression by modulating the extent of lipid peroxidation in leaves. They prevent lipid peroxidation by scavenging lipid peroxyl radicals and by reacting with other reactive oxygen species in cooperation with carotenoids. The antioxidant activity of vitamin E is supported by the ascorbic acid–glutathione (Asc-GSH) cycle. The cycle frees Tocopherl from the radical.

CHAPTER 2: MATERIALS AND METHODS

Study Goal and Justification

The goal of this study was to evaluate the effect of TRF on the lipid profile in chronic kidney patients on HD. The study justifications were based on the following:

1. Tocotrienols are known to have anti-inflammatory and anti-oxidant properties with potential lipid-modifying effects [53, 84, 86].
2. Most T3 studies were conducted on animals or cells. Khosla et al and Fairus et al studied the postprandial bioavailability of T3 in human [63, 65]. Qureshi et al found a lipid-lowering effect of T3 on humans [80, 81]. One study involved TRF and presented evidence that T3 may benefit lipid profile and improve apoprotein A1 expression (HDL) [94]. There are limited human studies evaluating T3 on patients on HD [51].
3. CKD is associated with dyslipidemia, inflammation and oxidative stress [12-14, 32]. T3 have more potent antioxidant and anti-inflammatory effects than tocopherols [51-53]. The current study was an attempt therefore to document the effect of TRF on inflammatory markers, oxidative stress and lipid profile in patients with ESRD who are under treatment by HD.

Patients

One hundred and eighteen patients were screened from the Great Lakes Dialysis Center, LLD (Detroit, Michigan). Study criteria included age (must be an adult no less than eighteen year old), duration of dialysis (at least three months), not living in a nursing home and receiving tube

feeding or intradialytic parenteral nutrition and not taking dietary supplements that contain vitamin E.

Study Design and Flow

A randomized, placebo controlled, double-blind parallel trial was conducted. The study was approved by the Human Investigation Committees of Wayne State University and the Human Investigation Committees of Great Lakes Dialysis, LLC. All patients signed an informed consent before the beginning of the study (Figure 2.1). The study lasted sixteen weeks.

A total of eighty eight patients (43 males and 38 females) were eligible and met the criteria. Patients were assigned to placebo (P) (n=40) and (TRF) (n=41). The average age of our patients was 59±12 year old with no significant differences between the placebo and the TRF groups. Average mass was 85±23 kg for placebo group and 90±26 kg for TRF group. There were 43 males (23 P and 20 TRF) and 38 females (17 P and 21 TRF). Eighty patients were from an African American background, and one was Caucasian. Fifty one patients were diabetics (25 P and 26 TRF).

Subjects were divided into TRF and placebo groups. Forty one subjects received two capsules of 110 mg/day of TRF supplements per day. Each palm TRF soft gel contained 90 mg tocotrienol isomers (34% α, 3% β, 50% γ and 13% δ) and 20 mg α-tocopherol. Forty subjects received placebo capsules made from wheat germ oil providing 0.24 mg tocotrienol and 0.44 mg tocopherol. Each subject received either 2 TRF capsules or 2 placebo capsules at the start of each dialysis, thrice weekly. For non-dialysis days, each patient was given a pill organizer that contained either TRF or placebo capsules. They were distributed on site at time of dialysis. Instructions were given to take the pills during lunch and dinner (the two main meals). Patients

returned the pill organizer and received a new refilled one every week. The remaining (non-used) pills were counted and tracked. Pill counting followed previously prescribed methodology [42, 97].

One patient from the placebo group suffered diarrhea during week one of the study, one patient from the TRF group was excluded after week one because of catheter dysfunction. One patient from the TRF group refused to continue after week four. Two patients from placebo group passed away from cardiac arrest (week three and week twelve).

Anthropometric measures were obtained at baseline, week-8, week-12 and week-16. The study was accompanied by 24-hour dietary recall during baseline (week zero) and week sixteen. Dietary intake was obtained by the same registered dietitian from all subjects.

Laboratory Assessments

Blood was drawn and collected at weeks zero (baseline), eight, twelve and sixteen. Blood collection tubes contained EDTA and lithium heparin (BD Franklin Lakes, NJ). Plasma samples were isolated by centrifugation at 2800 rpm for 20 minutes at 4°C. Plasma was divided into aliquots and stored at -80°C for further studies. Standard renal profiles such as serum albumin, blood urea nitrogen, kt/v (dialysis clearance per time/volume, and creatinine), cell blood count (CBC), hemoglobin and hematocrit were analyzed by an external laboratory (Satellite Laboratory Services, Redwood City, CA) using standard automated techniques.

Antioxidant capacity thiobarbituric acid reactive substances (TBARS) standard kit was used to measure malondialdehydes generated from oxidized lipids (MDA). Inflammatory markers Interleukin-6 (Il-6), C-reactive protein (CRP), tumor necrosis factor (TNF), and nuclear factor kappa B (NF-κB) were also measured.

Lipid profiles including plasma triglycerides (TG), total cholesterol (TC), and high density lipoprotein-cholesterol (HDL-C) were measured using enzymatic kits (Pointe Scientific Inc., Canton, MI). HDL-C was measured in the supernatant after precipitation of apoprotein-B containing lipoproteins by dextran sulfate and magnesium ions (Pointes Scientific Inc., Canton, MI). Plasma low density lipoprotein-cholesterol (LDL-C) was calculated using the Friedwald equation [LDL-C= TC – (HDL-C) – (TG/5)].

Cholesteryl ester transfer protein (CETP) activity in the plasma was measured using astandard kit as per manufacturer's protocol (BioVision, Mountain View, CA). Briefly, plasma samples were incubated at 37°C with a donor molecule containing a fluorescent self-quenched neutral lipid that was transferred to an acceptor molecule in the presence of CETP which resulted in an increase in fluorescence. Intensity of fluorescence was measured using fluorometer with excitation set at 465 nm; and emission at 535 nm (Tecan, Switzerland). CETP activity was quantified and expressed as picomoles per microliter of plasma per hour. Apo-protein A1 (apoA1) was measured using a double antibody sandwich ELISA method and followed the protocol described by the manufacturer. (Immunology Consultants Lab Inc., Portland, OR).

Microsoft office word 2007 and Microsoft Office Excel 2007 were used for data processing and graphing. Statistical analysis was obtained using SPSS (v16; IBM, Chicago, IL). Mean differences of the two groups were tested with independent t-test. Data were obtained using mean±standard. Difference between two time-points such as difference between baseline and week 12 was calculated using parallel t-test. Correlation tests were calculated using Pearson's correlation test. P values of < 0.05 was statistically significant.

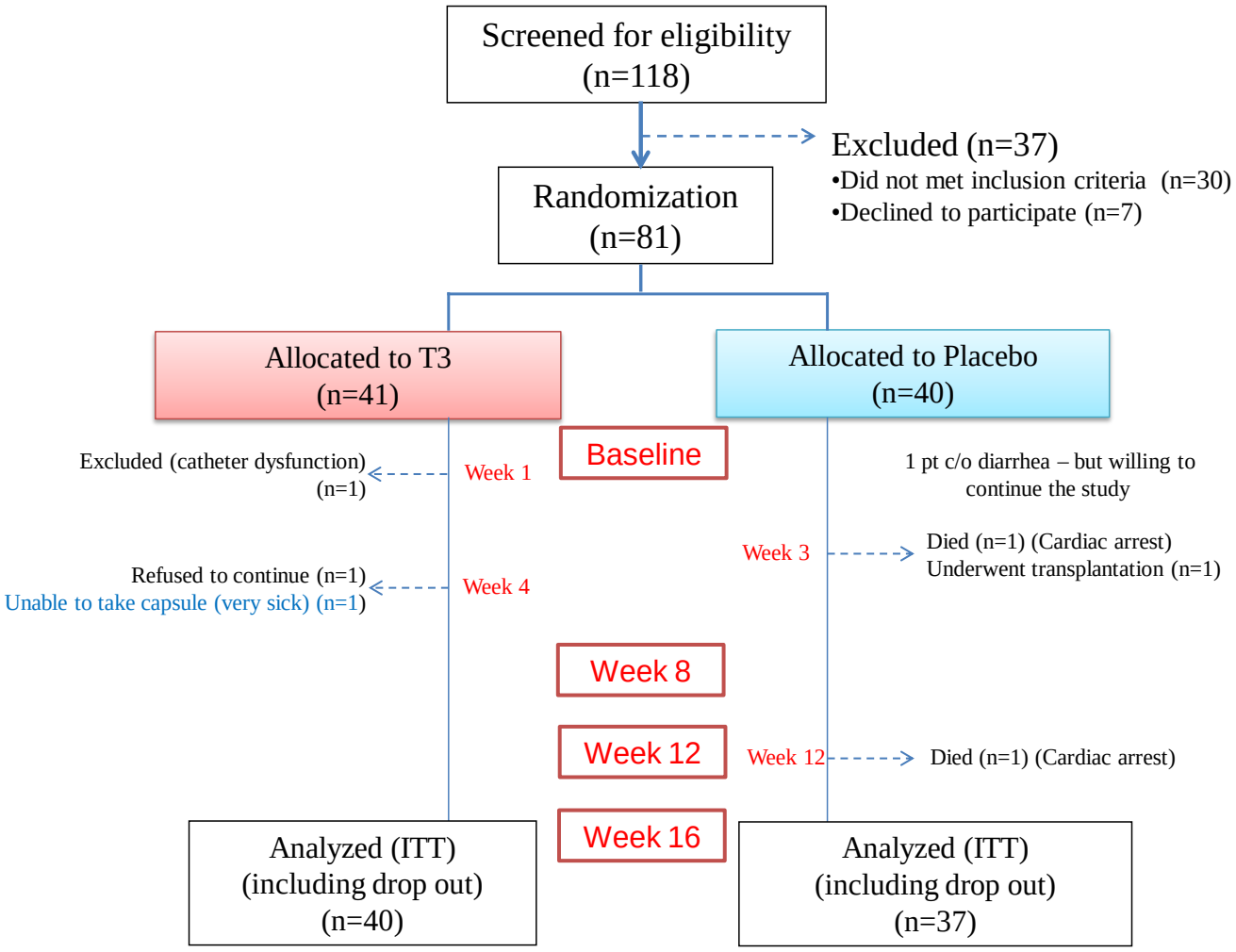


Figure 2.1: Flow chart of the study.

(May Daud, 2013)

CHAPTER 3: RESULTS AND DISCUSSION

Lipid Analysis

Blood samples were collected from 40 P and 40 TRF on week zero (one patient was excluded because of catheter infection), P=36, TRF= 39 on week 8, P =34, TFR= 38 on week 12 and P =29, TFR= 38 on week 16. All lipid analyses' quantitative results are presented in Table 3.1.

Plasma TC had a baseline average 183 mg/dl in TRF group and 179 mg/dl in P group. Both levels were reduced during week eight and twelve. By week sixteen, TC had an average of 145 mg/dl in TRF group and 150 mg/dl in P group. The independent t-test statistical analysis of total plasma cholesterol did not show significant difference between the two groups.

Total plasma TG was higher among the TRF group at the initial start (average 153 mg/dl TRF vs. 110 mg/dl P). Throughout the study, TG level remained higher among the TRF group. There was a reduction in TG to an average of 104 mg/dl TRF vs. 96 mg/dl Placebo in week sixteen. The decline of TG level during week twelve and sixteen was more noticeable in the TRF group because it revealed a sharper decline by about 50 mg/dl in TRF as compared to 14 mg/dl in P from baseline. We normalized plasma TG because there was significant difference at starting TG values between TRF and P group (Figure 3.1).

Total plasma HDL-C had a baseline average 42 mg/dl in TRF group and 44 mg/dl in P group. There was a statistically significant increase in HDL-C during week twelve among the TRF group (65 mg/dl) compared to 55 mg/dl in the placebo. HDL-C remained higher among the TRF in week sixteen (Figure 3.4). We normalized HDL-C for further analysis and that revealed an increase among TRF group at week twelve and sixteen (Figure 3.2).

Total plasma LDL-C revealed a gradual reduction in LDL-C among both groups with results of 75 mg/dl among P as compared to 66 mg/dl among TRF in week sixteen. Independent t-test did not show a statistical significance.

Plasma TC/HDL-C baseline ratio was higher in the TRF group than that of P group. It declined among the TRF group during the latter two weeks (2.30 week twelve and 2.60 week sixteen among TRF as compared to 2.80 and 2.81 among P). Normalization of TC/HDL-C ratio showed decline of TC/HDL-C ratios in TRF group at week 12 and 16 (Figure 3.3).

Inflammatory markers were non-significant (excluding the baseline of NFκ-B with a p=0.004). We also observed changes in oxidation in total antioxidant capacity week twelve with a p value of 0.027. There may be several reasons that explain what we found. 1) Some patients may have received a lower dose of T3 per weight. 2) HD and the co morbidities may modify metabolism and duration of TRF among patients.

Newly born VLDL is released from the liver in order to transport endogenous lipids. VLDL acquires apoprotein E (apo E) and apoprotein C (apo C) from HDL. Apo E aids in attaching VLDL particles to the cells of the endothelium the liver. Apoprotein CII (apo CII) activates lipoprotein lipase (LPL) while apo CI and CIII inhibits it [98]. VLDL releases TG under the effect of LPL into the intimae of the small vessels of muscles, adipose cells and the heart. The rest converts to intermediate-density lipoprotein (IDL) and subsequently to LDL. LDL is rich in cholesterol and low in TG. LDL delivers cholesterol to tissues for synthesis and maintenance [19].

Numerous studies found that ESRD and HD patients have impaired clearance of VLDL, which leads to its accumulation and further vulnerability to oxidative stress. The impaired clearance results in a delay in its catabolism [12, 32, 98], which is associated with dissemination

of oxidative stress and circulating oxidized lipids [39]. A decrease of apo C-II/ apo C-III ratio also leads to more inhibition of LPL [32]. LDL resulting from abnormal catabolism of VLDL tends to have structural abnormalities such as the presence of small dense LDL particles [98, 99]. HD patients have similar LDL-C level findings to those with CKD. There remains controversial whether small LDL articles tend to be more atherogenic than those larger articles [100]. There is decreased level of LDL receptors and expression of cholesterol clearance from the liver among patients with ESRD [72, 104]. It is the quantitative rather than the qualitative alterations of LDL that becomes troublesome. The same studies also found that LDL-C levels may be lower among some HD patients, but tend to be higher in those with nephrotic syndrome [32, 98]. Our data revealed that LDL-C levels did not express significant changes between the P and the TRF groups.

There is a deregulation of lipoprotein receptors and their regulatory transcription factors and enzymes (SREBP1 and 2 and HMGCR) in patients with HD and ESRD [38, 101, 102]. It contributed to an increased level of TG among HD patients [101, 102]. The fact that TRF group has a lower normalized TG levels would imply a potentially suppressive role of TRF on these regulators. This potentially includes those of upstream regulators of lipid homeostasis (SREBP 1/2, HMGCR, APB100, DGAT2), which would require future studies [103]. Such abnormalities also lead to an increase in uptake of oxidized lipids and lipoprotein into arterial and renal tissues and is also mediated by increased levels of pro-inflammatory cytokines.

The increase of plasma HDL-C among TRF groups prompted us to analyze the function of CETP in order to explain the activity of TG-cholesterol ester (CE) exchange between apo B carriers (VLDL and LDL) and apo A1 carrier (HDL). Numerous studies revealed that CETP has dual effects on lipid metabolism. It can act as pro-atherogenic because it increases CE transfer to

apo B-containing protein, small LDL-C and modifies HDL-C. It also act as anti-atherogenic factor because of its effect on enhancing LDL-receptors and LCAT which is an enzyme involved in reverse cholesterol transfer (HDL formation) [104, 105].

The role of HDL is to remove excess cholesterol from extra hepatic tissues. These tissues are unable to metabolize cholesterol [19, 106]. The major apolipoprtein in HDL is apo A1. It is secreted from the liver to the plasma in a low-lipid form. Then, lipids are added to the inside to produce a complete HDL molecule. The first step is to transfer phospholipids and unsterilized cholesterol from tissues to generate the nascent HDL (discoid). This step is mediated by ATP binding cassette transporter A-1 (ABCA 1) [19]. The discoid HDL piles up unesterified cholesterol from other lipoproteins. Apo A-1 activates several enzymes including lecithin cholesterol acyltransferace (LCAT) which causes the esterification of free cholesterol inside HDL and changes HDL form to a spheroid. Spheroid HDL is rich in cholesterol esters in its center. Apo A-1 and apo A-II (which is also synthesized in the liver) bind together and add to the structure of the spherical apo A-1 and apo A-2 rich HDL [19, 83, 106].

The liver picks up cholesterol from HDL via scavenger receptor B1 (SR-B1) and transform it into bile salts for excretion. The rate-limiting enzyme is a cytochrome p-450 mediated oxidation of cholesterol known as cholesterol 7 α hydroxylase. Apoprotein A1 is also catabolized by the kidneys. Lipid-poor apo A-1 are filtered at the level of the glomerulus and then catabolized by the proximal tubules [83]. The protein cubilin binds HDL Apo A-1 at a great affinity, interacts with a receptor megalin (related to a low-density lipoprotein receptor families). Cubilin is also the endocytic receptor for intrinsic factor for vitamin B12 [107]. This leads to uptake and catabolism (degradation) of apo A1 [107]. The rate-limiting step of apo A-1 clearance is the glomerular filtration level which explains the increase in apo A-1 and catabolic rate in

patients with ESRD [107]. Another pathway to transfer lipids from HDL is carried out by hepatic lipase (HL) which hydrolyzes triglycerides (TG) and phospholipids (PL) from HDL [83]. When HDL is rich in TG, this mechanism kicks in at the hepatocytes. This process is also mediated by an increase in CETP activity and mediation to exchange between HDL-cholesterol and TG from apo-B rich lipoprotein. The exchange results in an increase in TG in HDL and increase in cholesterol in apo-B containing lipoprotein which increase the risk of atherogenesis [19, 83, 106]. CETP and HL favor a small sized and less stable HDL including the generation of HDL3, β HDL and apo A-1 [108-110]. Thus CETP does not only exchange cholesterol and triglycerides, but also alter the size and the function of HDL and release apo A1 [111].

An increase in HDL-C among TRF group may be caused by delayed HDL catabolism or higher cholesterol levels in HDL, which resulted from decrease or alteration in CETP activity. Our data showed significant increase in apo A1 level among TRF group during week twelve. We saw higher plasma apoA1 level on week twelve among the TRF group (Figure 3.4). This increase corresponds with the increase of HDL-C in TRF group in week twelve because apo A1 is the main apoprotein in HDL molecule [83, 108]. The increase of apo A1 among TRF group warrants further studies because effects of T3 on apo A1 in patients with HD is not well studied. We speculate possible effects on transcription factors such as PPARα and PPARγ, which are involved in pro-apoprotein A1 [112].

Individuals with IR (many HD patients have IR or DM) are known to have an increased rate of apo A-1 catabolism and an increase in hepatic lipase activity [83]. The study by Chee Heng et al, 2012 also found that apo A1 expression is higher among individuals who received TRF among both young and old individuals. This study was not done on ESRD patients [47].

Individuals with IR and CKD tend to have decreased HDL level due to an increase in the fractional catabolic rate of apo A-1. Apo A-1 catabolic rate is increased in dialysis patients [108].

The higher level of HDL-C and apo A1 in TRF (week twelve) can not only be justified by the failure of its clearance due to ESRD and HD. We would see the same increase in the P group if that was the case. Alteration of CETP activity may thus explain alteration of TG-cholesterol shuttle between apo A1 and apo B containing lipoproteins (HDL and VLDL/LDL, respectively). We saw an increase of CETP activity at week twelve followed by reduction of CETP activity among TRF group in week sixteen (Figure 3.5). This goes with the elevation of HDL-C in week twelve among the TRF group. Our data showed an increase in normalized HDL-C and a reduction in normalized TG among the TRF group in both weeks. We conducted a correlation test between CETP activity and TG as well as between HDL-C and apo A1 during weeks twelve and sixteen to confirm. There was a positive correlation between CETP activity and TG concentration (Figures 3.6 and 3.7) and between HDL and Apo A1 (Figure 3.8 and 3.9). Correlation studies answered our speculation and confirmed our findings.

Table 3.1: Lipid profiles

	Baseline			**Week 8**			**Week 12**			**Week 16**		
	Placebo (n=40)	TRF (n=41)	P Value	Placebo (n=36)	TRF (n=39)	P Value	Placebo (n=34)	TRF (n=38)	P Value	Placebo (n=29)	TRF (n=38)	P Value
TC (mg/dl)	178±42	183±49	NS	153±32	157±36	NS	140±31	141±43	NS	148±37	145±44	NS
TG (mg/dl)	109±63	153±21	NS	105±52	139±86	**0.04**	100±57	113±47	NS	95±48	103±44	NS
HDL-C (mg/dl)	44±12	42±13	NS	51±14	51±15	NS	53±13	63±15	**0.009**	54±11.8	58±18	NS
LDL-C (mg/dl)	112±38	110±48	NS	80±31	76±35	NS	70±31	56±38	NS	75±33	66±42	NS
TC/HDL-C ratio	4.20±1.30	4.61±1.72	NS	3.13±1.08	3.20±1.05	NS	2.80±1.01	2.31±0.80	**0.046**	2.81±1.01	2.60±1.02	NS

Abbreviations: TC=Total cholesterol, TG=Triglycerides, HDL-C=High density lipoprotein cholesterol, LDL-C= Low density lipoprotein cholesterol.

Note: Data are expressed as mean±standard deviation. P values are derived from independent t-test.

Plasma lipids (total cholesterol (TC), total triglycerides (TG), HDL-C, LDL-C, and TC/HDL-C) for week zero, eight, twelve and sixteen. All values are presented as mean ± SD. P values were derived using an independent t-test, which tested mean differences between lipid profiles in the TRF and placebo groups.

P values were derived using an independent t-test, which tested mean differences between lipid parameters in the TRF and placebo groups with a significance when $P < 0.05$. NS indicates non-significance.

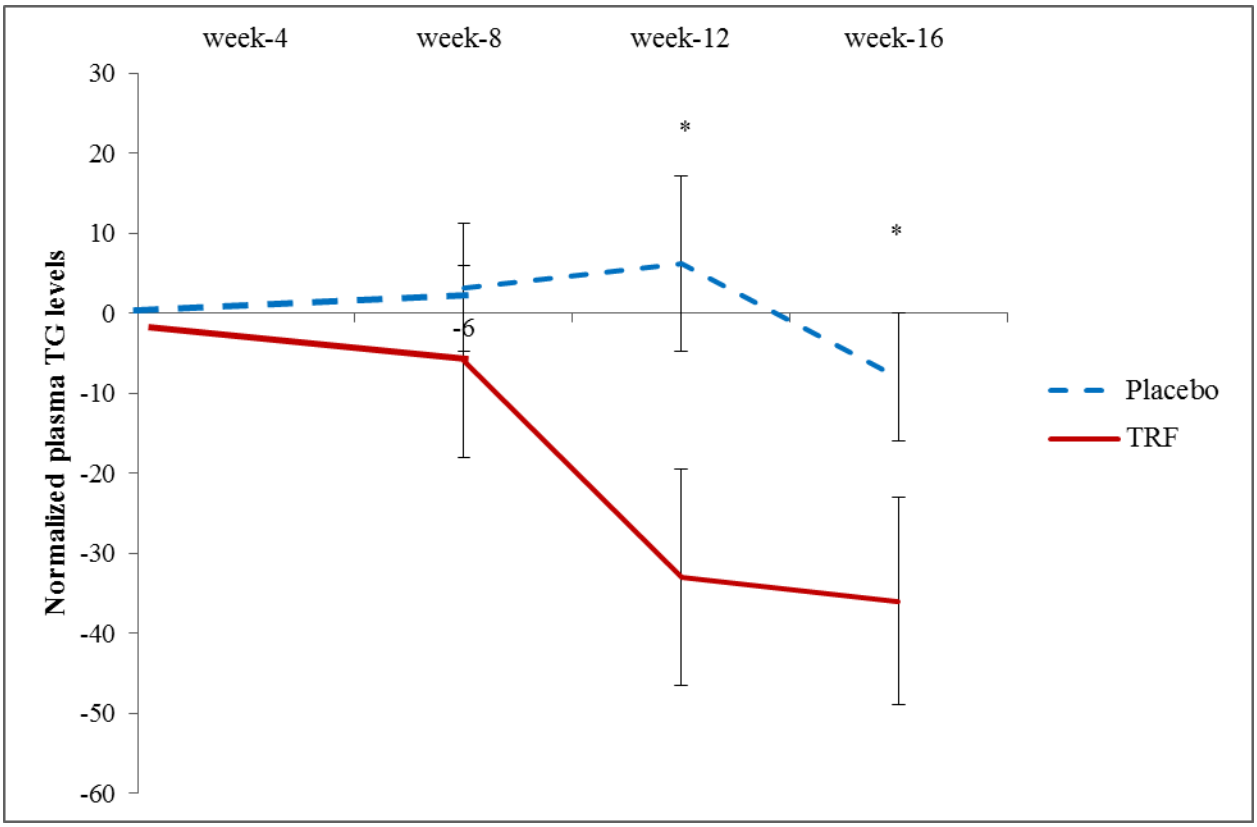


Figure 3.1: Normalized plasma triglycerides week 8, 12 and 16 mg/dl. Patients were grouped into P and TRF. Values were presented as mean ± SD.

P values were derived using repeated measures ANOVA with post hoc Bonferroni test, which tested mean differences in lipid parameters within the groups over the time course. Mean difference between TRF and placebo groups at particular time points were tested using independent t-test. P value < 0.05 is considered significance.

The asterices indicate significance ($p < 0.05$).

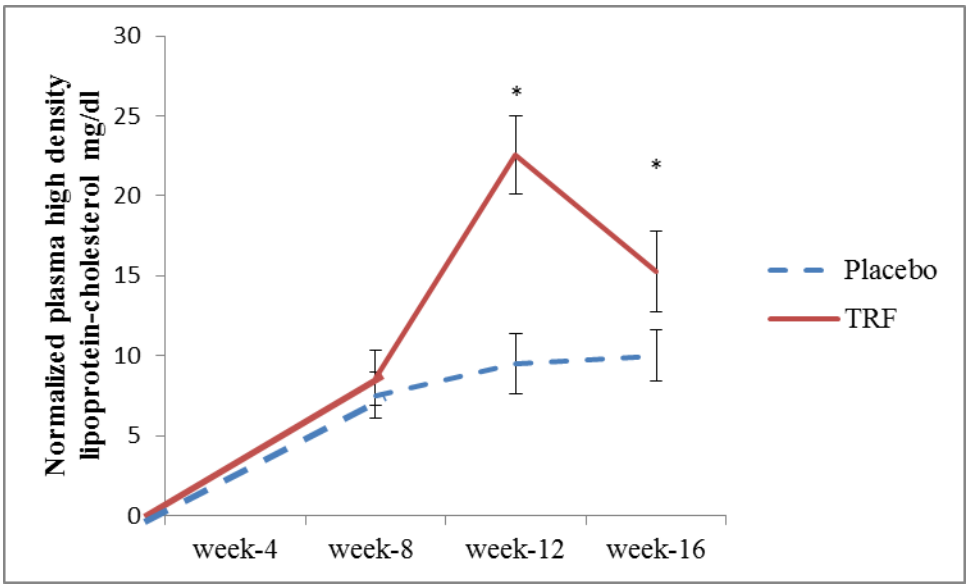


Figure 3.2: Normalized plasma high density lipoprotein-cholesterol mg/dl week 8, 12 and 16 mg/dl. Patients were grouped into P and TRF. Values were presented as mean ± SD.

P values were derived using repeated measures ANOVA with post hoc Bonferroni test, which tested mean differences in lipid parameters within the groups over the time course. Mean difference between TRF and placebo groups at particular time points were tested using independent t-test. P value < 0.05 is considered significance.

The asterices indicate significance ($p< 0.05$).

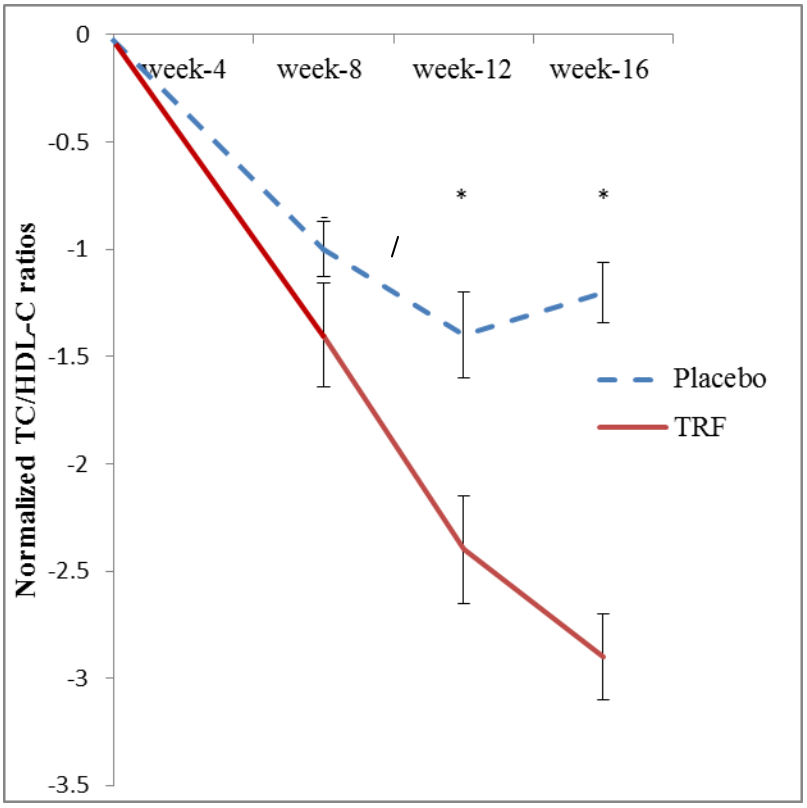
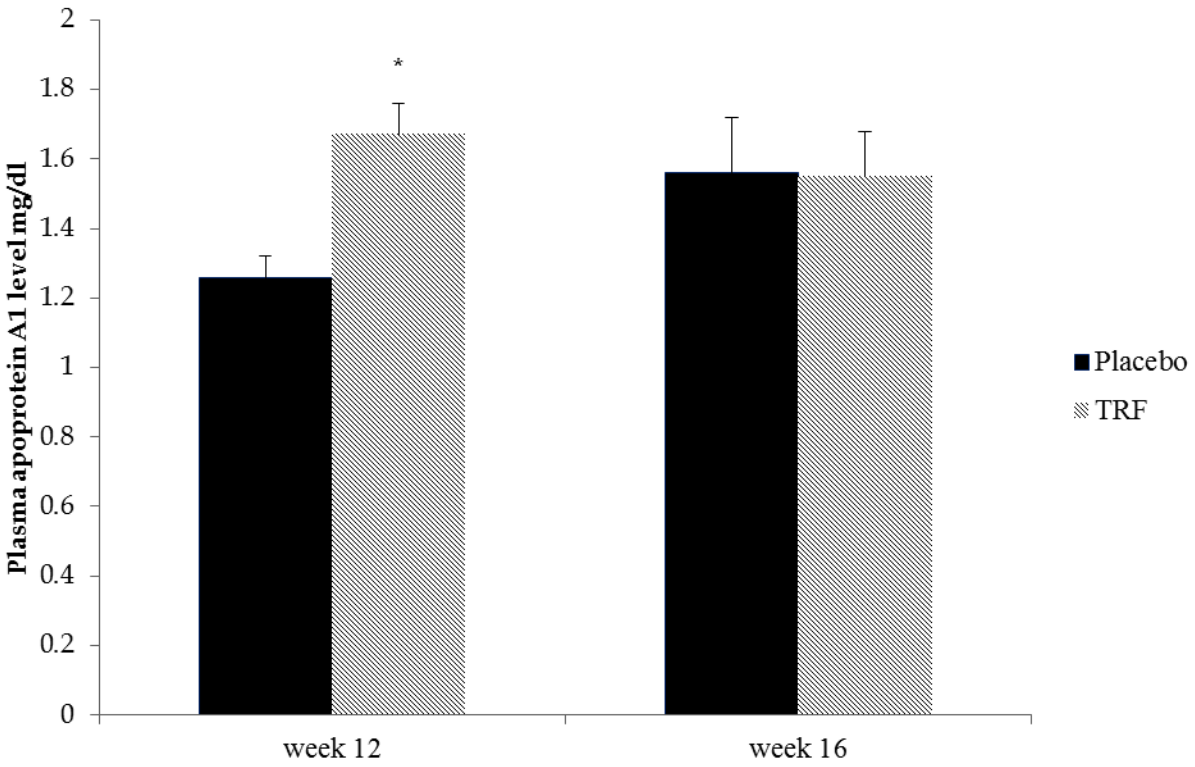


Figure 3.3: Normalized plasma TC/HDL-C ratios week 8, 12 and 16 mg/dl. Patients were grouped into P and TRF. Values were presented as mean ± SD.

P values were derived using repeated measures ANOVA with post hoc Bonferroni test, which tested mean differences in lipid parameters within the groups over the time course. Mean difference between TRF and placebo groups at particular time points were tested using independent t-test. P value < 0.05 is considered significance.

The asterices indicate significance ($p < 0.05$).



FFIGURE 3.4: Plasma apoprotein A1 measures mg/dl in P and TRF groups. Values were presented as mean ± SME. Number of initial participants was 35 P and 39 TRF at week 12 and 34 P and 38 TRF and week 16.

P values were derived using an independent t-test, which tested mean differences between lipid parameters in the TRF and placebo groups with a significance when $P < 0.05$.

The asterix indicates significance ($p< 0.05$).

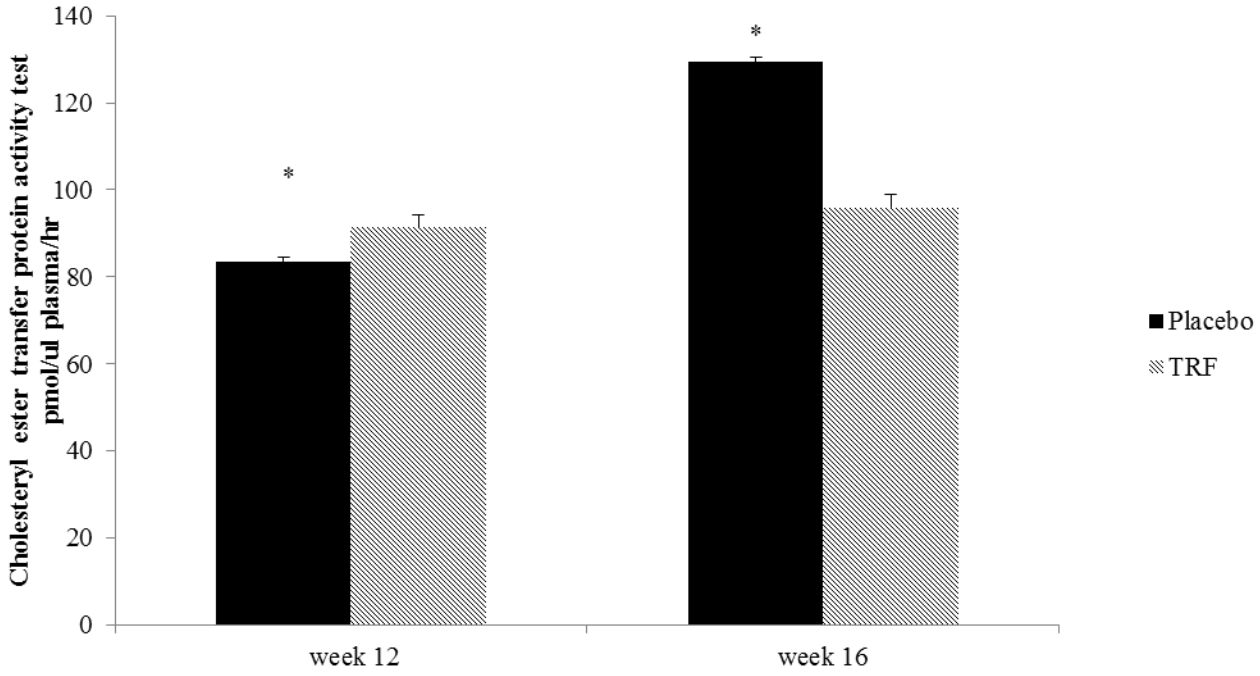


FIGURE 3.5: Cholesteryl ester transfer protein activity test (pmol/ ul plasma/hr) in placebo and TRF groups. Values were presented as mean ± SME. Number of initial participants was 35 P and 39 TRF at week 12 and 34 P and 38 TRF and week 16.

P values were derived using an independent t-test, which tested mean differences between lipid parameters in the TRF and placebo groups with a significance when $P < 0.05$.

The asterices indicate significance ($p< 0.05$).

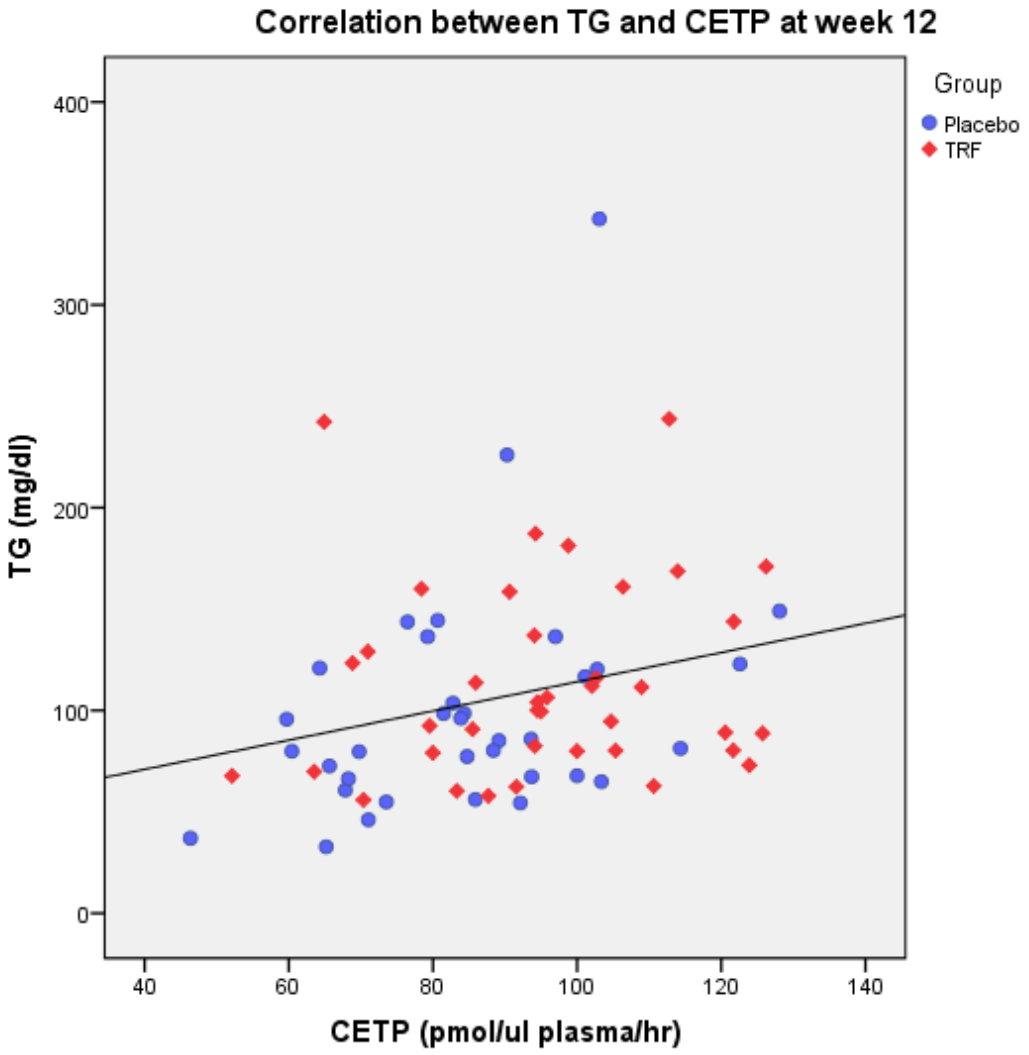


Figure 3.6: Correlation between plasma TG (mg/dl) and CETP activity (pmol/ ul plasma/hr) for week 12. Pearson correlation was done for statistical analysis with r=0.260, p= 0.025.

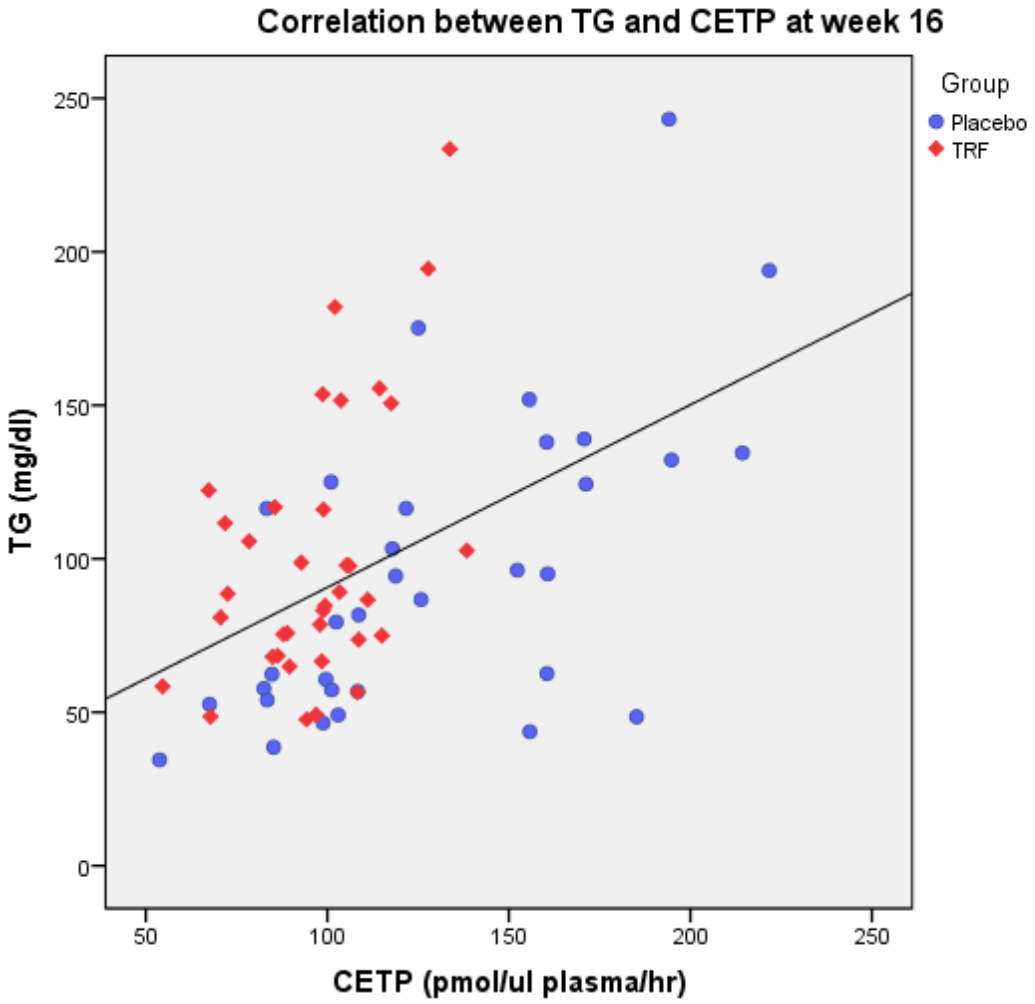


Figure 3.7: Correlation between plasma TG (mg/dl) and CETP activity (pmol/ ul plasma/hr) for week 16. Pearson correlation was done for statistical analysis with r=0.475, p= 0.000.

Correlation between HDL-C and Apo A1 (week 12)

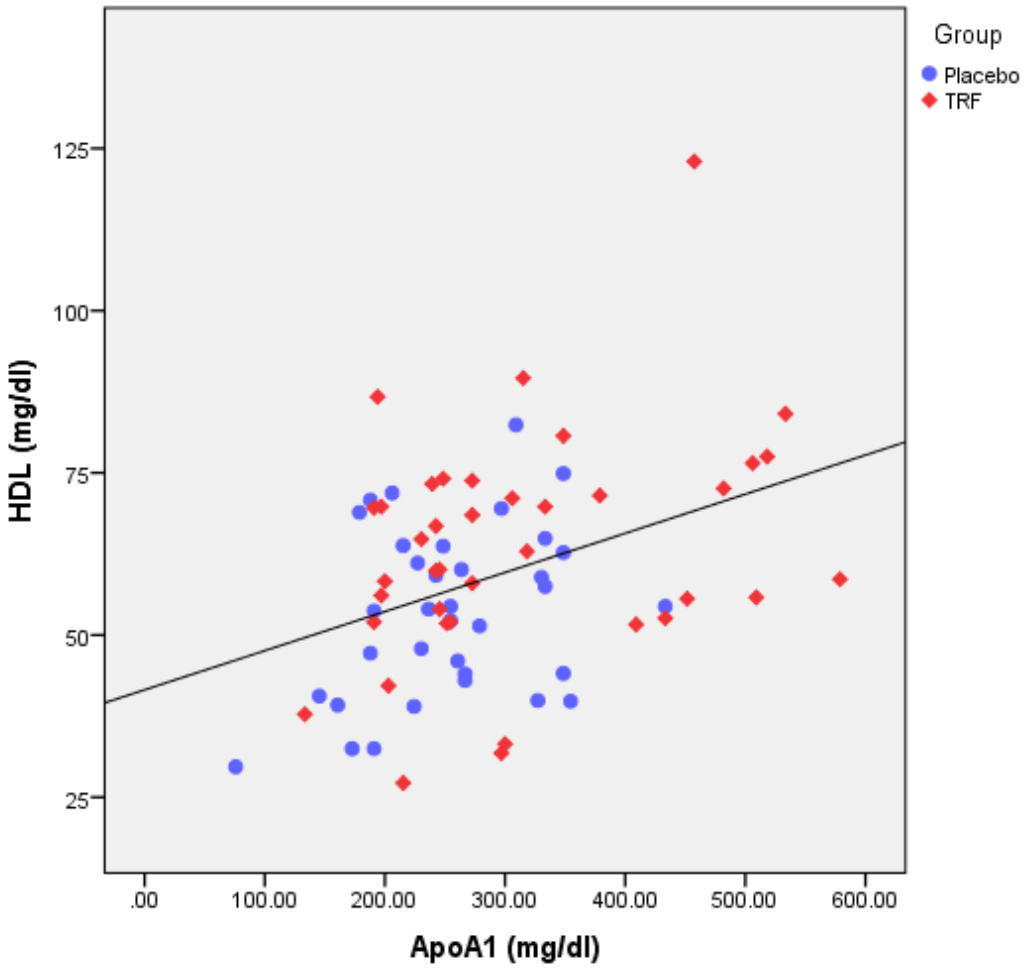


Figure 3.8: Correlation between HDL (mg/dl) and apoA1 (mg/dl) for week 12. Pearson correlation was done for statistical analysis with r=0.378, p= 0.001.

Correlation between HDL-C and Apo A1 (week 16)

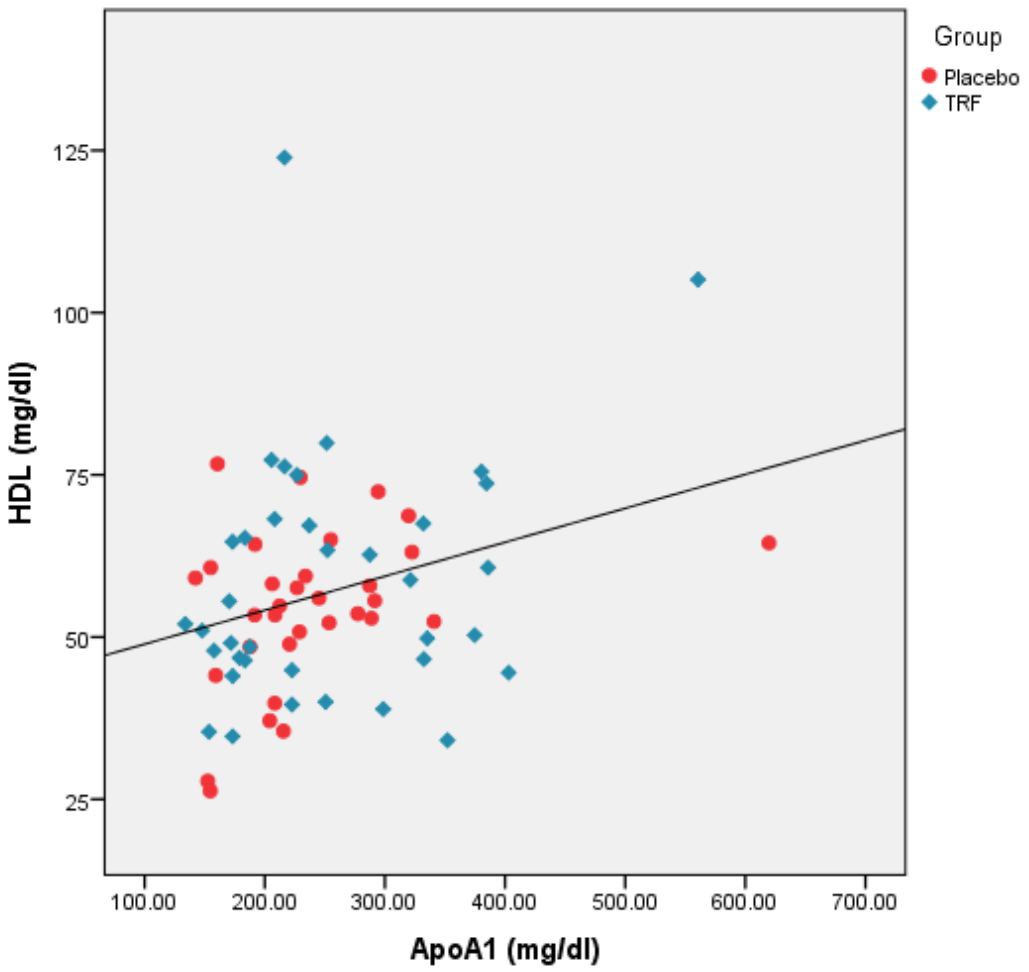


Figure 3.9: Correlation between HDL (mg/dl) and apoA1 (mg/dl) for week 16. Pearson correlation was done for statistical analysis with r=0.295, p= 0.014.

CHAPTER 4: SUMMARY AND CONCLUSION

CKD and ESRD are characterized by changes in blood level of lipoprotein, their sizes, and their function. The alterations in the enzymes that carry on the inter-change between cholesterol and triglycerides including CETP play major roles along with deficiencies in receptors removal [12, 32, 39, 98, 108]. Numerous studies found that CKD and uremia do not only cause low HDL, elevated TG and alteration of CETP activity in HD patients, but also alter HDL composition and function [113, 114].

The elevated level of TG among HD patients (both P and TRF groups) might be explained by dysfunctional clearance of VLDL and lower function of HL [38, 98,108]. Studies also revealed that HDL in CKD and HD patients has different proteonomic composition [114]. IR and other co morbidities also would lead to such metabolic picture of these patients. Many of our patients have diabetes. Advanced DM and metabolic syndrome are major risk factors for CKD and IR is also associated with dyslipidemia and CKD complications [16, 21]. We found an increase in plasma HDL-C and reduction in plasma TG among the TRF patients during week twelve and sixteen. Normalization revealed a statistically significant increase of HDL-C and reduction of TG in the TRF group (week 12 and 16). We also found a reduction in TC/HDL-C among TRF patients because HDL increased. Our data would imply a lipid lowering or modifying effect of TRF, which requires future studies.

An attempt was made to explain the changes in HDL-C and TG metabolism by measuring apo-A1, which is the major apoprotein found in HDL and by measuring CETP activity, which is a major enzyme involved in cholesterol-TG shuttle between apo-B and apo A1 lipoproteins.

We found positive correlation between HDL-C and ApoA1 (weeks 12, 16; respectively), which ought to be further explained based on the understanding of HDL metabolism. The enzyme CETP transfers TG from apo-B lipoprotein to apo-A (HDL subtypes). CETP also leads to remodeling of the size and the structure of HDL [39, 98, 114]. The transformation of spherical HDL to a smaller size HDL is carried on by CETP [105, 111, 113]. HDL subunits (apo A1 and apo A1/A2) are also altered in patients with CKD which explains their altered cardio protective activity [114]. Apo-A1 catabolism and clearance is also reduced because of the alteration of its renal clearance in HD patients [83, 108]. The increase of free apo- A1 among TRF group in week twelve mirrored the increase in HDL-C [109]. If the increase in apo A1 among TRF at week twelve was only caused by lack of renal clearance because of HD and the fact the kidneys catabolize apo-A1, we would have seen elevation in apo A1 in both groups and throughout the study. This was not the case.

We measured CETP activity, and there was higher activity of CETP in the P group (week twelve), while CETP activity in the TRF group showed reduction at week sixteen. We conducted a correlation study between CETP and TG (suggesting TG activity transfer) and between HDL-C and apo A1 in week twelve and sixteen to verify if there was consistency. Correlation studies were positive in both weeks confirming our findings. The data suggested a potential effect of TRF on CETP activity in the TRF group that would explain the elevation of HDL-C, apo A1 and the reduction of TG.

We concluded that TRF might have reduced CETP activity, lowered TG and raised HDL-C and apo A1. We concluded that TRF is potentially beneficial for HD patients because our data showed improvement in lipid profile in TRF group (lowering TG and increasing HDL-C).

Further studies

Further studies may be required in order to verify and illicit the mechanism(s) which TRF affect lipid profile in HD patients. This requires a larger number of patients and a potential increase in the study duration or the dose of T3 based on their tolerance, co-morbidities and drug treatments. A multi-center research study is thus required to conduct research and assess the time-course and the potential improvement with giving a higher or modified dose of TRF on human patients with ESRD with HD and PD. The goal is to elicit the effect of TRF, the effect of each of the four individual TRF isomers or the comparison between TRF isomers and those of tocopherols and/or with other neutraceuticals including antioxidants and fish oil may be warranted. The following studies are suggested:

A) To conduct genomic-proteonomic studies eliciting the effect of TRF on lipid transcriptional regulation, synthesis, and receptors in patients with ESRD with and without HD or PD. B) To conduct a study eliciting the effect of TRF on metabolism of various apo-As and apo B lipoproteins and various regulatory lipoprotein enzymes such as CETP in patients with ESRD. C) To further elicit the effect of T3 on the associated co-morbidities and their interaction with the other medical (lipid lowering drugs, ant diabetics, antihypertensive, etc.) and nutritional interactions on patients with ESRD with HD or PD in order to elicit better understanding and clinical approach.

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ABSTRACT

EFFECTS OF TOCOTRIENOL RICH FRACTIONS ON LIPID PROFILES IN HEMODIALYSIS PATIENTS

by

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Chronic hemodialysis (HD) patients have an increased risk of cardio vascular diseases (CVD) driven by dyslipidemia, oxidative stress and inflammation. Vitamin E isomers (tochopherols and tocotrienols) are fat-soluble anti oxidants. Tocotrienol isomers (T3) are fewer studies than tochopherol isomers, but they have multifaceted effects on oxidative stress, inflammation and lipid metabolism.

We investigated the lipid modifying effects of tocotrienol rich fractions (TRF) on CKD patients receiving HD in a randomized, placebo-controlled, double-blind parallel trial on 81 patients (43M, 38F). Subjects (n=41) were given 220 mg/day of either TRF (180mg TRF, comprising of 34% αT3, 3%βT3, 50% γT3, 13%δT3 and 40 mg α- tocopherol), or placebo (n=40) which provided 0.24 mg T3 and 0.44 mg α-tocopherol.

We used standard kits to measure lipid profile=[plasma triglycerides (TG), plasma total cholesterol (TC), HDL-cholesterol (HDL-C), LDL- cholesterol (LDL-C) using Friedwald equation, and the corresponding TC/HDL-C ratios] during stating point, week 8, week 12 and week 16. Statistical analysis used double t-test and for mean differences between TRF and

placebo groups with $p<0.05$ as significant. We found that TG was progressively declined in the TRF group while HDL-C improved at week 12 and 16($p<0.05$). We evaluated CETP activity and apo A1 using a fluoremetric essay and Elisa, respectively to further evaluate HDL and apo B lipoprotein metabolism.

We found an increase in plasma apo A1 among TRF group at week 12 as compared with placebo, while week 16 changes were attributed to depressed CETP activity. We concluded that TRF supplements improved lipids in this HD group. A multi-center trial is needed to further study the mechanism which TRF work, and with higher doses of TRF.

AUTOBIOGRAPHICAL STATEMENT

The author received his doctor of medicine (MD) from Tishreen University of Lattakia, Syria. He subsequently immigrated to Canada and obtained bachelor's degrees in art history, mathematics and education from the University of Windsor, Canada. He taught for several years before starting his master's degree in nutrition at Wayne State University. He also taught nutrition courses at Wayne State University.