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# Effects Of Palm Oil Tocotrienols Rich Fraction On Cholesterol Efflux

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# EFFECTS OF PALM OIL TOCOTRIENOLS RICH FRACTION ON CHOLESTEROL EFFLUX

by

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## DEDICATION

"To my parents, husband, family members and all those who supported me throughout my academic life"

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| | |
|---|---|
| CVD | Cardiovascular diseases |
| LDL | Law density lipoproteins |
| HDL | High density lipoproteins |
| FBS | Fetal Bovine Serum |
| PBS | Phosphate Buffered Saline |
| Apo-A | Apolipoprotein A |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| SRA1 | Scavenger Receptor A1 |
| CD-36 | Cluster Determinant 36 |
| SRB1 | Scavenger Receptor class B type 1 |
| LXR-α | Liver-X receptor alpha |
| ABCG1 | ATP Binding Cassette Subfamily G member 1 |
| ABCA1 | ATP Binding Cassette Subfamily A Member 1 |
| PCR | Polymerase Chain Reaction |
| RNA | Ribonucleic acid |
| DNA | Deoxyribonucleic acid |
| cDNA | Complementary DNA |
| TRF | Tocotrienol Rich Fraction |
| NSCLC | Non-small cell lung cancer |
| Notch-1 | Notch-homolog 1 |

# CHAPTER 1: Introduction

## 1.1 Chronic Kidney Disease (CKD)

Chronic Kidney Disease (CKD) is a global epidemic which has become a huge burden to the healthcare system in the USA and countries worldwide. It has gained the global attention due to the rapid increase in prevalence, high cost in treatments, high risk associated with Cardiovascular disease (CVD) and need of effective therapies to prevent disease progression [1]. The National Kidney Foundation (NKF) reports that CKD affects approximately 10-13% of the world population and causes millions of deaths each year [2]. It is reported that among people aged from 65 to 74, every one in five men and one in four women suffers from CKD worldwide. According to NKF, CKD was the 27$^{th}$ cause of total number of deaths worldwide in 1990, but rose to 18$^{th}$ by 2010, indicating a high movement up the list. This high movement was only second to that of HIV and AIDS [3].

Among world CKD population, over 2 million receive treatment for CKD either through dialysis or kidney transplantation [4]. In the US, treatments for CKD utilized 6.7% of total medicare budget in 2010 and the cost is likely to exceed $ 48 billion per year [2, 3]. In China, the estimated cost for treatment for next decade is over $ 558 billion, whereas in Australia the estimated cost until 2020 is $ 12 billion [2]. The cost of CKD treatment in the UK exceeds the combined cost of skin, lung, breast and colon cancer treatments [2]. Furthermore, in Uruguay the annual cost of dialysis is nearly $ 23 million, which is 30% of the budget for National Resources Fund for special therapies [2]. In middle income countries, most CKD patients are unable to afford dialysis or kidney transplantation, which leads to death of over 1 million people annually due to untreated kidney failure in those countries [4]. In developing countries, the number of CKD patients is estimated to increase

due to the increasing number of elderly population [3]. Therefore, CKD has become a huge financial burden to a country's economy as well as to the patient.

### 1.1.1 Stages of CKD

The National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KIDOQI) guidelines characterize CKD into 5 stages, based on Glomerular Filtration Rate **(Table 1.1)** [5]. According to KIDOQI guidelines CKD is defined as kidney damage of 3 or more months duration caused by structural or functional abnormalities with or without a decreased GFR. Chronic kidney disease is also defined as the presence of kidney damage or GFR less than 60 mL/min/1.73 m2 for 3 months or longer, irrespective of cause [6].

**Table 1.1: Stages of CKD**

| CKD Stage | Description | GFR (ml/min/1.73 $m^2$) |
|---|---|---|
| 1 | Kidney damage with normal or ↑ GFR | > 90 |
| 2 | Kidney damage with mild ↓ in GFR | 60-89 |
| 3 | Moderate ↓ in GFR | 30-59 |
| 4 | Severe ↓ in GFR | 15-29 |
| 5 | Kidney failure | < 15 (or dialysis) |

Source: United States Renal Data System [5]

GFR at the stage 1 is >90 ml/min/1.73 $m^2$. At this stage kidneys have about 90-100% functionality and this stage can be determined using routine laboratory measurements [7]. Stage 2 is also known as mild CKD and at this stage kidneys have 60-89% functionality [7]. These patients may be asymptomatic but may have high blood pressure, urine abnormalities and normal or slightly elevated serum creatinine levels. Since early stages (stage 1-2) can be asymptomatic, development of CKD can only be determined using lab tests such as hemoglobin test, urine test for albumin and blood test for serum creatinine [7].

Decrease in hemoglobin level indicates decrease in erythropoietin production, which occurs due to altered kidney function. Presence of albumin in the urine or microalbuminuria, is another early sign of kidney disease [7].

If the disease progress to stage 3 and 4, patients may have elevated serum creatinine level with 30-59% and 15.29% kidney functionality respectively [9]. At these stages, all organs are affected with evident complications including anemia, fatigue, decreasing appetite with progressive malnutrition, abnormalities in calcium and phosphorus level. Progression to stage 5 causes kidney function to less than 15% and leads to accumulation of toxins [7]. Patients experience loss of urine production and are subjected to severe life threatening complications such as difficulty in breathing and chest pain due to fluid retention, severe weaknesses, coma and high level of potassium which could affect function of the heart [7]. This stage is also known as kidney failure or End Stage Renal Disease (ESRD) and these patients need dialysis or renal transplant to survive.

**1.2 Cardiovascular diseases (CVD) in CKD population**

CKD is a major risk factor for CVD and is one of the leading cause of death in CKD. Most CKD patients die due to CVD even before reaching ESRD [5]. According to United States Renal Data System (USRDS), CVD causes more than half of deaths with known causes in ESRD patients [5]. The two major CVD events that occur in ESRD patients are atherosclerotic heart disease (ATHD) and congestive heart failure (CHF). In CKD patients increased levels of oxidative stress, inflammation, atherogenic oxidation-prone lipoproteins and small dense LDL particles and qualitatively dysfunctional HDL particles are believed to accelerate atherosclerosis.

### 1.2.1 Atherosclerosis

Atherosclerosis consists of series of events that occur in the arterial wall, which leads to narrowing of arteries through formation of a fatty streak. This process initiates as a result of endothelial dysfunction that occurs due to prolonged exposure to hypertension, hyperlipidemia, toxins or smoking which can activate endothelial cells [8]. Activated endothelial cells secrete vasoconstrictor factors which cause constriction of arteries, factors that affect growth and differentiation of vascular smooth muscle cells and expression of cellular adhesion molecules [9]. Through these adhesion molecules, monocytes are attracted and attached to the endothelial surface and enter the intima, where they differentiated into macrophages. These macrophages engulf oxidized LDL which have entered the intima via endothelial injury, and become foam cells.

As this process continues, foam cells secrete growth factors that stimulate migration of smooth muscle cells into the intima. These smooth muscle cells interact with foam cells to form fatty streak, which is then converted to advanced lesions. Although initial stages such as foam cell formation can be reversible, prolonged exposure to risk factors promote formation of an atheromatous plaque, that can protrude into the lumen, leading to narrowing the artery [9, 10] **(Figure 1.1)**.

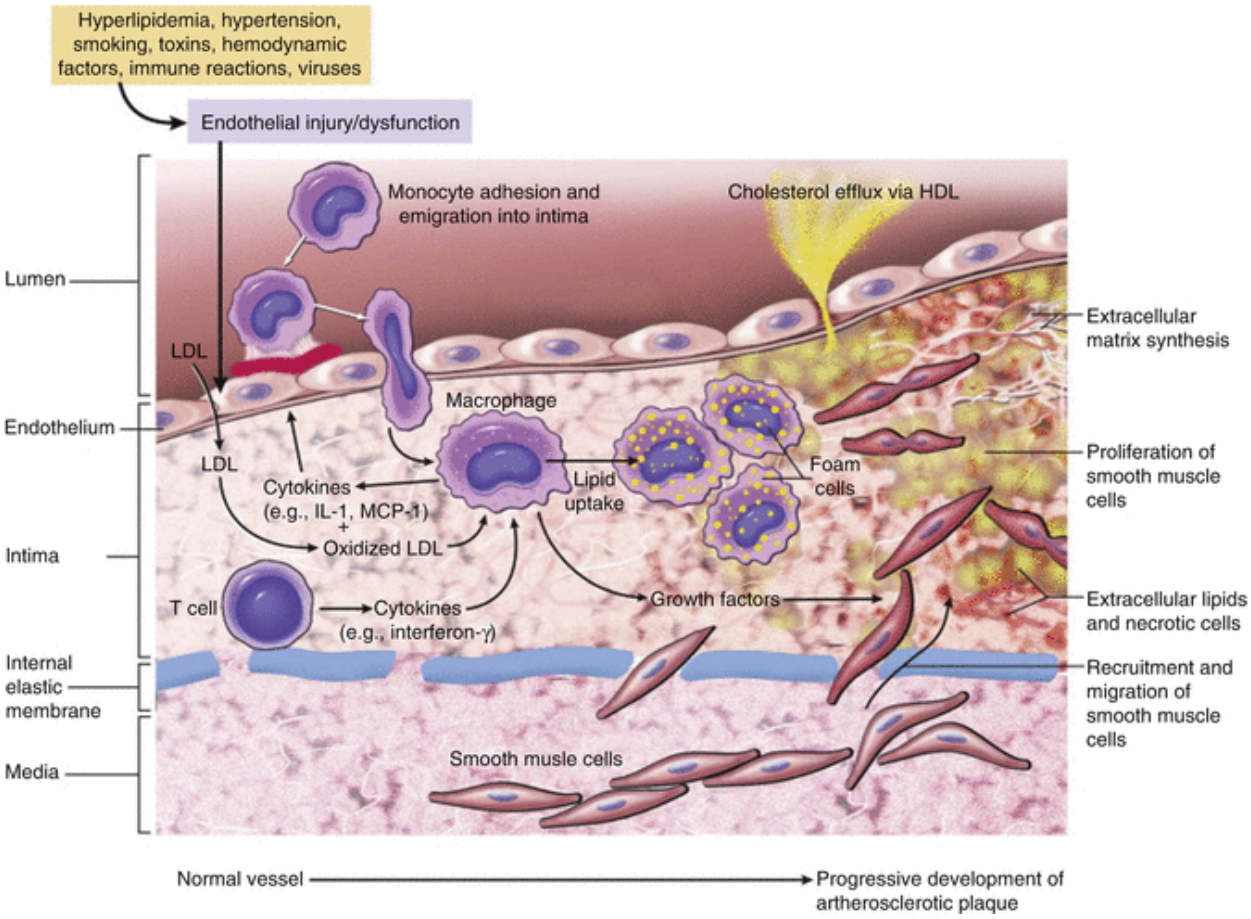



**Figure 1.1: Formation of atheromatous plaque**

(Source: Pathophysiology of Coronary Artery Disease [10])

### 1.2.2 Causes of Atherosclerosis

**Oxidative stress**

Reactive oxygen species (ROS) such as superoxide and peroxide radicals are formed during energy production in the cells by mitochondria. In this process, free electrons are transported through electron transport chain to its final acceptor, molecular oxygen. However, some electrons may escape the chain, forming ROS such as superoxide radicals and peroxide radicals [11, 12]. The level of ROS in the body is balanced by the body's

antioxidant level and as the level of ROS over anti-oxidant level increases, it causes oxidative stress. ROS are highly reactive and can rapidly interact with DNA, proteins and lipids in the cells. Unsaturated lipids such as polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) which present in cell membranes, neurons and LDL particles can be readily oxidized by ROS. Lipid radicals and peroxyl radicals formed by this process are highly reactive and can produce more free radicals via interaction with other molecules and cellular compartments **(Figure 1.2)**. Thus, increased oxidative stress in the body can increase the level of oxidized LDL in plasma which can promote atherosclerosis.

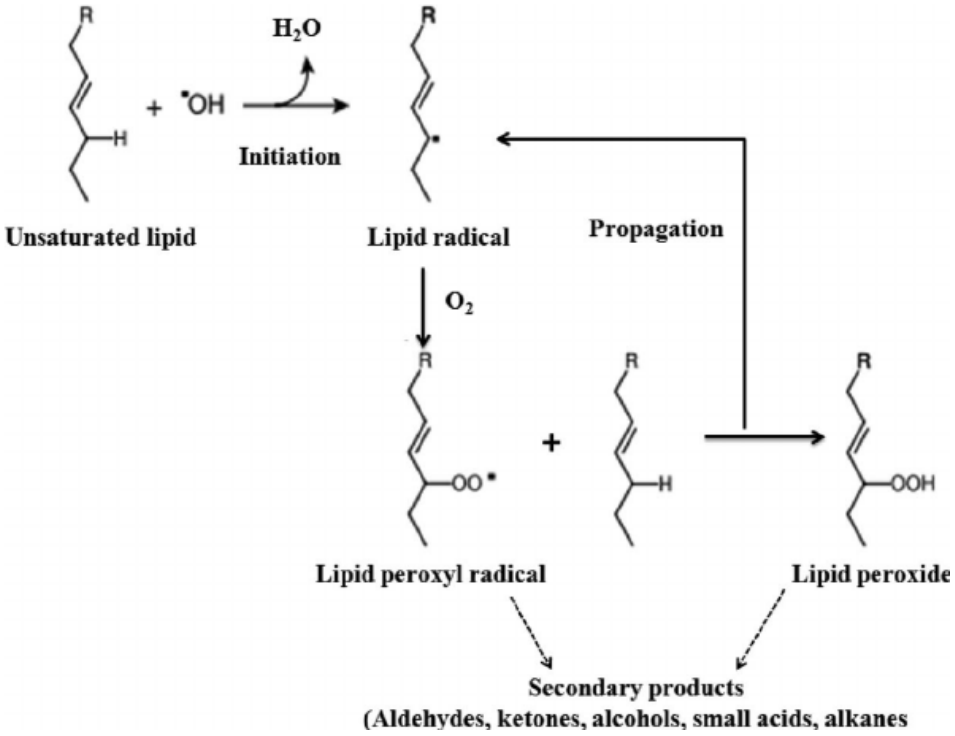



**Figure 1.2: Lipid peroxidation**

**(**Source: Jayasena et al [12])

**Inflammation**

Inflammation participates in atherosclerosis from its early stages. Diets high in saturated fat, hypercholesterolemia, obesity, hyperglycemia, insulin resistance, hypertension, and smoking, release pro-inflammatory signals which stimulate arterial cells to produce adhesion molecules, that initiate early stages in atherosclerosis [13, 14]. Inflammation is known to cause qualitative alterations in LDL particles, which make them highly susceptible for oxidation [13]. A higher level of oxidized LDL in the plasma, is known to stimulate NFκB pathway, that triggers the production of pro-inflammatory cytokines which promotes foam cell formation [13]. Hyperglycemic conditions seen in type-2 diabetes is known to increase the production of advanced glycation end products (AGE) which have pro-inflammatory properties and stimulate pro-inflammatory cytokines production from adipose tissue [13, 14]. In addition, decreased plasma HDL level and anti-oxidant enzymes associated with HDL such as paraoxonases, may also promote inflammation.

**Altered lipid metabolism**

One of the leading causes of atherosclerosis is abnormalities in lipid metabolism. Elevated small dense LDL particle level, lipoprotein (a) (Lp-a), decreased endothelium bound LPL, upregulated HMG-CoA reductase and Acyl-Co A: cholesterolacyltransferase (ACAT) activity are reported to promote atherosclerosis [8]. Small dense LDL particles are considered to be a marker for carotid atherosclerosis as they can enter the arterial intima through infiltrating the vessel wall [8]. While remain trapped inside the intima, small dense LDL are highly susceptible to oxidative modification which can promote foam cell formation [8].

Lp-a is a modified lipoprotein which is degraded by kidney. Therefore, in CKD, Lp-a level may increase and accumulate in the atherosclerotic lesions. As a result, elevated level of Lp-a is considered as an independent risk factor for CVD in CKD patients. Also in CKD patients, decreased endothelium bound lipoprotein lipase (LPL) levels are also observed which can decrease the clearance of LDL and increase accumulation of LDL in blood [15]. These processes increase plasma LDL level, which triggers LDL oxidation and foam cell formation, in the presence of high oxidative stress. In addition, CKD impairs metabolism of high density lipoproteins (HDL) and reverse cholesterol transport pathway (RCT), which play critical roles in removing excess cholesterol from peripheral tissues.

### 1.3 Reverse Cholesterol Transport (RCT)

Cholesterol homeostasis in the body is maintained by balancing plasma cholesterol inflow to plasma cholesterol outflow. In cholesterol metabolism, peripheral cells receive cholesterol through apo B containing lipoproteins such as LDL, which are enriched with cholesterol derived from either diet or endogenous synthesis [15]. Excess cholesterol from peripheral tissues including arterial wall, are removed by a RCT **(Figure 1.3)** [15, 16]. RCT can be mainly divided into several steps namely,

(1) cholesterol efflux from macrophages

(2) transport of cholesterol in to plasma

(3) uptake by the liver and

(4) excretion from the body

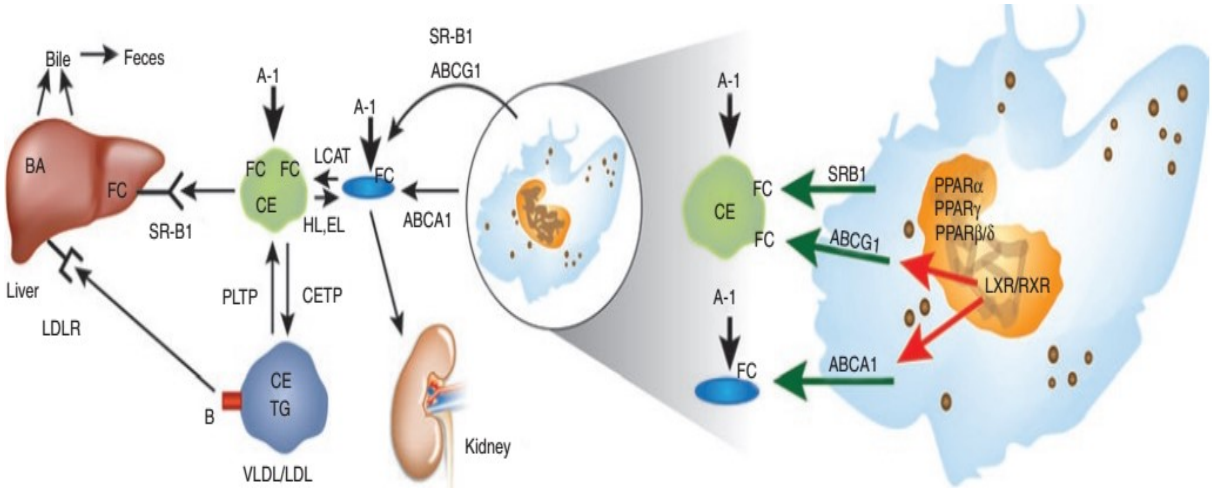



**Figure 1.3 Reverse Cholesterol Transport**

(Source: Gutstein et al [16])

### 1.3.1 Cholesterol efflux

Cholesterol efflux is first step in RCT and involves up taking oxidized cholesterol in the arterial intima, into the macrophages and releasing excess cholesterol into HDL particles. Altered cholesterol efflux causes accumulation of excess cholesterol in macrophages, leading to foam cell formation which initiates atherosclerosis [17, 18].

The uptake of modified LDL occurs through cholesterol influx receptors such as CD 36 and SRA1. These two receptors are known to be 90% responsible for uptake of oxidized cholesterol into macrophages [19]. Once taken into macrophages, oxidized LDL are catabolized in the lysosomes in the macrophages. Cholesterol esters in LDL particles are then broken down to free cholesterol and fatty acids by lysosomal acid lipase (LAL) [19]. Free cholesterol is then converted to cholesterol esters by acyl coenzyme A: cholesterl acyltransferase (ACAT 1) in the endoplasmic reticulum (ER) in the macrophage. A secondary hydrolysis of cholesterol esters to cholesterol takes place through neutral

cholesteryl ester hydrolases, and free cholesterol liberated are effluxed from macrophages through cholesterol ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 and scavenger receptor SR-BI [8, 20]. Free cholesterol is mainly accepted from ABCG1 and SRB1 into large mature HDL (HDL-2) particles whereas Apo-A1 and small nascent HDL (HDL-3) particles are the main acceptors for free cholesterol from ABCA1 [8].

**1.3.2 Transporting cholesterol by HDL to liver**

Upon receiving cholesterol into small nascent HDL particles (HDL-3), HDL-3 activates lecithin-cholesterol acyltransferase (LCAT), which is an enzyme that converts cholesterol into cholesterol esters. This clears the surface space in small nascent HDL (HDL-3) particle to accept more free cholesterol and convert it into much larger spherical HDL (HDL-2) particles [8]. Cholesterol esters in HDL-2 particles are then exchanged to triglycerides in LDL/VLDL particles through Cholesteryl Ester Transfer Protein (CETP). HDL-2 particles rich with cholesterol and triglycerides are then taken up by Scavenger receptors class B type 1 (SRB1) which also present in hepatocytes in liver. In the liver, cholesterol transported by HDL-2 is de-esterified and secreted into the bile and excrete with feces.

In CKD, RCT is affected at several stages. Patients with CKD are reported to have significantly low plasma levels of cholesterol acceptors such as Apo-AI and HDL level in the plasma [8]. The low levels of HDL in these patients can be attributed to higher level of CETP [8]. CKD patients also known to have deficiency and reduced function of LCAT which impairs conversion of small dense HDL-3 into larger HDL-2. More importantly, HDL-3 are mainly degraded by the kidney [16]. Thus, in CKD accumulation of HDL-3 which has proinflammatory and pro-oxidative properties may occur. On the other hand,

due to reduced LCAT activity, CKD may reduce HDL-2 with anti-inflammatory and anti-oxidant properties [8]. Therefore, reduced HDL level in CKD, may increase the risk for atherosclerosis.

Although an increase in plasma HDL is considered to be atheroprotective, it doesn't necessarily reflect functional properties of HDL [8]. Dysfunctional HDL, even with a higher plasma level, may not uptake excess cholesterol, leading to a decrease in clearance of excess cholesterol from the body as bile. Therefore, a higher level of plasma HDL may not necessarily indicate protective effects from CVD [17]. Since cholesterol efflux require functional HDL, which can accept and deliver cholesterol, recent research suggests that cholesterol efflux, is a better predictor for CVD risk [17], compared to plasma HDL level. It has been suggested that an increased level of cholesterol efflux indicates reduced risk and protective effects from CVD and vice versa. Thus, current research focus on therapies which improve cholesterol efflux, as a prevention strategy for CVD.

### 1.3.3 Effect of PPAR-γ on cholesterol efflux

PPAR-γ acts as a key regulator of cholesterol influx genes such as SRA1, CD 36 and efflux genes such as ABCA1, ABCG1 and SRB1 [20-23]. Increased expression of PPAR-γ is known to increase an influx gene, CD 36, while decreasing the expression of another influx gene, SRA1 causing no net influx into the macrophages[22, 24, 25]. In addition, PPAR-γ is known to increase the expression of efflux genes such as ABCA1, ABCG1 and SRB1 [20]. Therefore, as a net effect, induction of PPAR-γ is believed to increase the net cholesterol efflux from the macrophages [21, 22, 24, 25]. Induction of ABCA1 and ABCG1

through PPAR-γ is known to occur through the induction of another gene, LXR-α, which is downstream to PPAR-γ but upstream to ABCA1 and ABCG1 **(figure 1.4)**[21] .

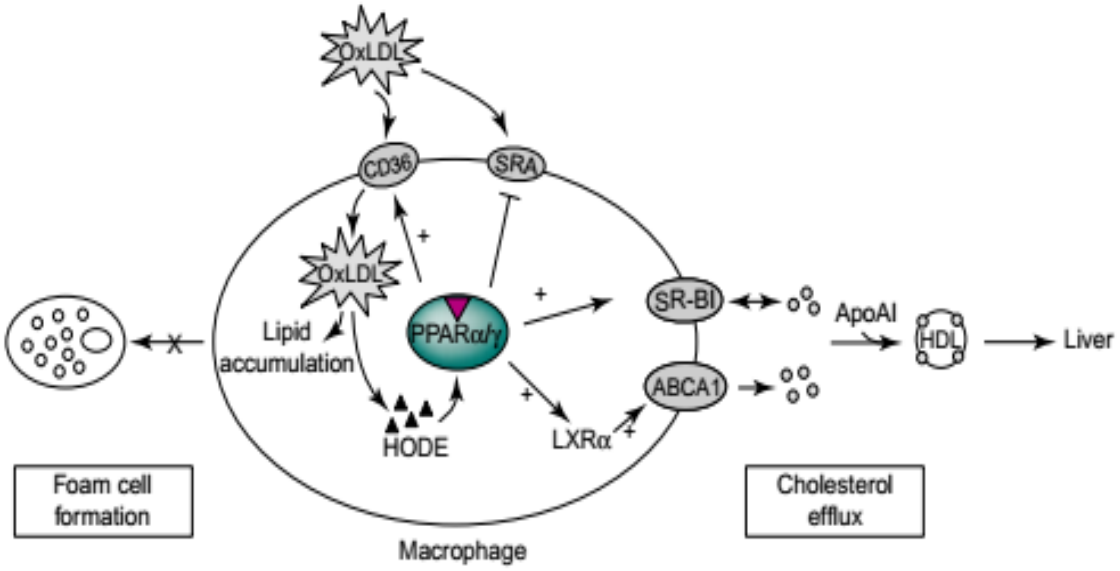



**Figure 1.4: Effects of PPAR-γ on cholesterol influx and efflux genes**

(Source: Duval et al [21])

Therefore, apart from dysfunctional HDL, altered expression of these genes can also lead to reduced cholesterol efflux.

Due to the interaction of PPAR-γ with influx and efflux genes, agents that can induce the expression of PPAR-γ can increase net cholesterol efflux leading to reduction in foam cell formation. Several research studies have reported the effect of wide variety of bioactive compounds and commercially available drugs as PPAR-γ agonists, in increasing cholesterol efflux [20, 24-28]. Anti-diabetic thiazolidinedione (TZD) drugs such as Troglitazone, have been shown to reduce foam cell formation through modulating PPAR-γ [24, 25]. Treatment with murine macrophages with TZD, induced PPAR-γ expression while down regulating SRA1 and upregulating CD 36, thereby causing no net effect on

cholesterol accumulation [24, 25]. Propofol, a phenolic derivative, was shown to increase SRB1 expression and ABCA1 expression through PPAR-γ-LXR-α pathway in THP-1 macrophages [27].

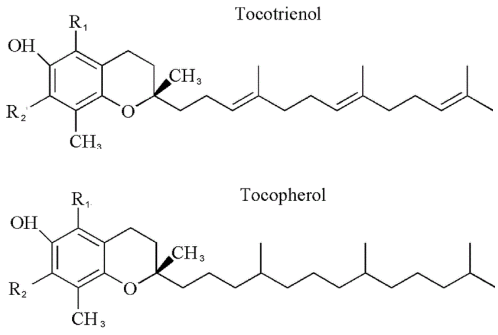
Due to the potential side effects associated with commercially available drugs, use of naturally occurring bioactive compounds in disease prevention and management has become a main focus of nutrition research. Several studies have reported the effect of bioactive compounds in cholesterol efflux [26, 29-32]. Ferrulic acid found in coffee, has been shown to increase cholesterol efflux via upregulation of SRB1 and ABCG1 mRNA and protein level in THP-1 human macrophages [33]. Resveratrol found in grapes, berries and walnuts was shown to increase LXR-α, ABCA1, ABCG1 and SRB1 mRNA expression level in THP-1 human macrophages[31]. In the same study, it was shown that treatment with resveratrol increased cholesterol efflux and suppressed foam cell formation. Resveratrol has also been shown to increase cholesterol efflux via an ABCA1- dependent manner and reduced cholesterol influx in J774A.1 macrophages[29]. In THP-1 macrophages, resveratrol increased ABCA1 expression via induction of LXR-α and reduced the expression of influx gene, SRA1 [34].

In a study performed with 13-Hydroxy Linolenic acid it was shown that Linolenic acid increases Apo-A1 mediated cholesterol efflux while increasing ABCA1, ABCG1 and SRB1 expression [35]. Furthermore, Curcumin an active compound in turmeric has been shown to increase ABCA1 gene expression while down regulating expression of SRA1 in J774A.1 macrophages [32]. In another study that used the same cell line, treatment with α-lipoic acid increased ABCA1 expression via induction of LXR-α [30]. In addition to these,

Vitamin E is also a potent bioactive compound which has been widely researched for its anti-oxidant, anti-cancer and anti-inflammatory properties [36].

### 1.4 Tocotrienols: A form of Vitamin E

Vitamin E was first discovered in 1922 and was extracted from alfalfa leaves to prevent placental hemorrhage in a rat model [37]. Two major isoforms of Vitamin E have been identified, namely tocopherols and tocotrienols. Each form is naturally available as chemically distinct isomers namely alpha (α), beta (β), gamma (ϒ) and delta (δ) making total number of vitamin E isomers into 8 **(Figure 1.5)** [38]. The structure of tocopherols compromises a chromanol ring and saturated hydrocarbon chain while tocotrienols have a similar chromanol ring but an unsaturated hydrocarbon chain with three double bonds [39]. Tocotrienols mainly found in palm oil, rice bran oil, barley germ and annatto. Palm oil has considerably higher amount of tocotrienols (940 mg/kg) and rice bran oil contains 465 mg/kg of tocotrienols [37]. In addition, tocotrienols are also found in olive oil, hazelnuts, maize, rye, flax seed oil, sunflower oil and grape fruit seed oil.




| isomer | $R_1$ | $R_2$ |
|---|---|---|
| α-tocopherol/tocotrienol | $CH_3$ | $CH_3$ |
| β-tocopherol/tocotrienol | $CH_3$ | H |
| γ- tocopherol/tocotrienol | H | $CH_3$ |
| δ-tocopherol/tocotrienol | H | H |

**Figure 1.5: Isomers of Vitamin E** (Source: Lu et al [38])

Being hydrophobic by nature, tocopherols and tocotrienols require an aid to penetrate the cell membrane. It has been found out that transport of tocotrienols and tocopherols into

cells occur via a special protein called tocopherol transfer protein (TTP) [36]. Although TTP has a higher affinity for tocopherols compared to tocotrienols, the unsaturation side chain of tocotrienols is known to facilitate better penetration of tocotrienols into fatty layers such as brain and liver [37, 40]. Also, tocotrienols are believed to incorporate and transfer between cell membranes more efficiently than tocopherols [41]. In addition, tocotrienols are believed to have a better distribution in the cell membranes and are known to accumulate in endothelial cells, 10 times higher than tocopherols which may account for its higher anti-oxidant, anti-cancer, hypocholesterolemic properties compared to tocopherols [42, 43].

Upon consumption, tocotrienols are cleaved by an esterase located in the small intestine and absorbed together with fat with the help of bile acids [37]. Absorbed tocotrienols are then packaged into chylomicrons and enter the lymphatic system. In the liver and peripheral tissues, they are digested by lipoprotein lipase that release constituents of lipoproteins, which then enter the tissues via receptor mediated endocytosis. In the body, highest amounts of tocotrienols are found in adipose tissue and adrenal gland [37].

### 1.4.1 Properties of tocotrienols.

Attention towards beneficial effects of tocotrienols arose in 1980's and 1990's when their hypocholesterolemic and anti-cancer properties were first discovered [37]. To date, a large number of research studies have been conducted to explore the anti-oxidative, anti-inflammatory, hypocholesterolemic and anti-cancer properties of tocotrienols and to understand how these properties may provide protective effects on chronic diseases [36].

**Anti-oxidative properties:**

The oxidation of biological molecules such as lipids, proteins and DNA by ROS, is a leading cause of numerous pathological events, including, but not limited to CKD cancer and aging processes. The anti-oxidant function of tocotrienols is known to occur through its strong free radical scavenging activity, which breaks the free radical chain reaction. More interestingly, tocotrienol radical formed in this process is resonance stabilized due to the conjugated system of the chromanol ring in tocotrienols. These tocotrienol radicals are converted back to tocotrienols by the vitamin C and glutathione reductase system **(Figure 1.6)** [44].

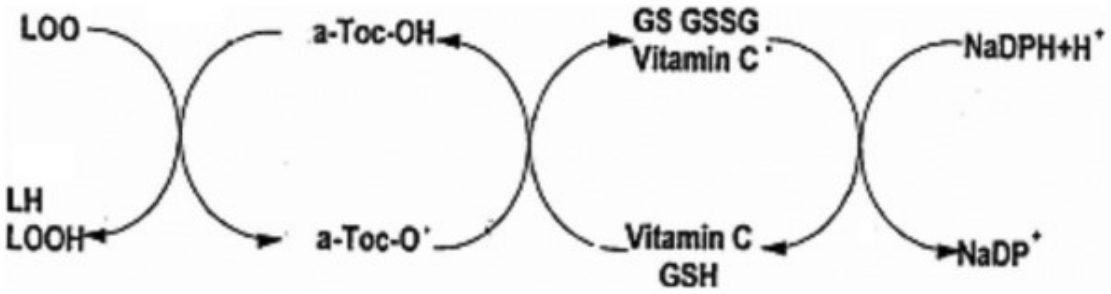



**Figure 1.6: Anti-oxidative properties of tocotrienols**

(Source: El-Beltagi et al [44])

Apart from scavenging free radicals, tocotrienols are known to exert its anti-oxidative functions through modulating enzymes in anti-oxidant system such as glutathione S-transferase (GST). In cultured rat hepatocytes treatment with tocotrienols and tocopherols together significantly decreased GST level in 3 days [37, 42]. In the same cell culture model, tocotrienol alone significantly decreased GST level in 1-3 days, with more prominent effect seen at concentration above 50 μM, compared to control and tocopherol [42].

In a Streptozotocin (STZ) induced diabetic nephropathy rat model treatment with tocotrienols (100 mg/kg) for 8 weeks, significantly reduced TABARs, a lipid peroxidation product, compared to control group [43]. In addition, treatment with a combination of insulin and tocotrienol significantly reduce the increase in TBARS compares to rats treated with insulin and tocotrienol alone [37].

**Anti-diabetic properties:**

Diabetes is the leading cause of CKD and 30-40 % of patents with type I diabetes and 15% of patients with type II diabetes develop ESRD. Tocotrienols have shown anti diabetic properties in different models. In Streptozotocin (STZ) induced diabetic rat model, treatment with tocotrienols (100mg/kg) for 8 weeks significantly decreased, superoxide dismutase and catalase enzyme activity compared to the control group. The effects seen in this model with tocotrienols are more prominent compared to effects seen with α-tocopherol [43]. Treating STZ induced diabetic rat model with α-Tocotrienol (0.1 g/kg) significantly prevented increase in advanced glycation end products (AGE), MDA and blood glucose level [45]. Furthermore, intragastric supplementation of palm of TRF (200mg/kg) significantly reduced the blood glucose level and glycated hemoglobin level in STZ-induced diabetic rats [46].

**Anti-inflammatory properties:**

CKD patients experience higher level of inflammation compared to a healthy population. Anti-inflammatory properties of tocotrienols have been tested on both cell culture and animal models, mostly using Lipopolysaccharide (LPS), a compound that is widely used to induce inflammation in cell culture experiments. In a study by Yam et al, induction with

LPS increased the expression of various inflammatory markers such as TRF-α, IL-6, COX-1, COX-2 in RAW.247 macrophages and incubation with 10 μg/ml of delta -tocotrienols for 24 hours significantly reduced the expression of these inflammatory makers [47]. In addition, treatment with LPS induced THP-1 macrophages with tocotrienols (0.5, 1.0 and 5.0 μg/ml) significantly and dose dependently reduced proinflammatory cytokines such as TRF-α, IL-4 and IL-8 and expression of NFκB [48]. In rats with STZ induced diabetic nephropathy, tocotrienols significantly and dose dependently decreased expression of NFκB, TNF-α and TGF-β1 which are increased due to STZ. It was found out that anti-inflammatory effect seen with tocotrienol is more prominent than tocopherol alone [43].

**Hypolipidemic properties:**

Tocotrienols also have shown hypolipidemic properties in both animals and humans. Ability of Tocotrienols to reduce plasma cholesterol level is reported to mediated by reducing HMG-CoA reductase activity, the rate limiting enzyme in cholesterol synthesis [37]. In another clinical study, supplementation of tocotrienols (75 mg/day) for 2 months, significantly reduced fasting blood lipid levels (31). In this study, total cholesterol (TC) and LDL-cholesterol (LDL-C) were dropped by 13% and 9-15% respectively, whereas high density lipoprotein-cholesterol (HDL-C) was increased by 4-7% at the end of the treatment [37]. In STZ induced diabetic rat model, treatment with TRF (200 mg/Kg body weight) for 8 weeks, significantly decreased plasma total cholesterol level, TG level, LDL level and significantly increased plasma HDL level compared to untreated group [46]. In a pre-clinical study conducted by our lab, hemodialysis patients supplemented with tocotrienols (two soft gels containing 90 mg of tocotrienols and 20 mg of α-tocopherols

per day) for 16 weeks, had significant reduction in plasma triglycerides levels and significant increase in plasma HDL levels, compared to placebo group [49].

**Anti-cancer properties:**

Tocotrienols have shown beneficial effects on different types of cancers such as lung, prostate, breast, colon cancer cell models [36]. Among these, Non-small cell lung cancer (NSCLC) is one of the leading cause of cancer deaths worldwide accounting for 20% of all cancer deaths and 80% for all lung cancer events [50]. Effects of tocotrienols on NSCLC have been extensively studied by Gupta lab in our department [50-52]. Based on their findings, δ-tocotrienols have shown to inhibit cancer cell proliferation, migration, invasion and induce apoptosis in NSCL cancer cell lines such as A549 and H1299 [50-52]. According to the Ph.D dissertation by L. Rajasinghe [53] similar effects are seen with TRF derived from palm oil. In these studies, they found out that tocotrienols exert its anti-cancer effects via inhibiting Notch-1 signaling pathway which involves cell proliferation, migration and invasion of NSCL cancer cells [50-53].

### 1.4.2 Effects of tocotrienols on RCT

Increase in reverse cholesterol transport is considered to reduce the risk for atherosclerosis. Based on recent studies, tocotrienols have affect several important enzymes and genes involve in RCT [49, 54]. In ApoE deficient mice, tocotrienols have shown to reduce atherosclerosis via inducing PPAR-γ and its down-stream genes such as LXR-α, suggesting tocotrienols can act as potential PPAR-γ agonists in reducing atherosclerosis [54]. Since PPAR-γ is a key regulator in cholesterol efflux, increase in PPAR-γ due to tocotrienols, may increase cholesterol efflux, which can reduce foam cell formation.

### 1.4.3 Clinical intervention study: Effects of TRF in hemodialysis population

Patients with ESRD have an increased risk for atherosclerosis due to elevated oxidative stress, inflammation and altered lipid metabolism [8]. In a randomized, double blinded, placebo controlled, clinical intervention study conducted by our lab, we found out that TRF supplementation significantly improved lipid profile in hemodialysis population. Eighty-one hemodialysis patients participated in this study and patients were given vitamin E tocotrienol-rich fraction (TRF) (180 mg tocotrienols, 40 mg tocopherols) or placebo (0.48 mg tocotrienols, 0.88 mg tocopherols) for 16 weeks [49]. Based on our findings, supplementing ESRD patients with TRF capsules for 16 weeks, showed increase in normalized plasma HDL (of baseline) at both 12 and 16 weeks compared to placebo. Similarly, TRF supplementation decreased normalized plasma TG (of baseline) at both 12 and 16 weeks compared to placebo. In addition, at 12 and 16 weeks, TRF supplementation showed increase in plasma Apo-A1 levels and decreased plasma CETP activity compared to placebo and baseline [49]. Decreased CETP activity and increased cholesterol acceptors such as HDL and Apo-A1 suggest that TRF may have beneficial effects in promoting reverse cholesterol transport in these patients.

Using the same capsules used in our pre-clinical study, Rajasinghe et al explored potential anti-cancer properties of TRF on NSCLC invitro. Based on their unpublished data, TRF dose dependently inhibited NSCLC proliferation, migration, tumor cell invasion and induce apoptosis through down regulating Notch-1 and NFκB pathways[*]. Findings of

[*] Rajasinghe, L. and S. Gupta, *Tocotrienols suppress non-small lung cancer cells via downregulation of the Notch-1 signaling pathway (644.1).* The FASEB Journal, 2014. **28**(1 Supplement): p. 644.1

These invitro studies further validate the multiple health benefits associated with TRF such as anti-cancer effects.

**Objective:**

In the pilot study conducted by our lab, we found out that TRF derived from palm oil increased both plasma HDL and Apo-A1 and reduced CETP in ESRD patients. The increase in HDL and Apo-A1 and decrease in CETP activity observed in patients supplemented with TRF may promote RCT, which can increase removal of excess cholesterol from the body. Apart from affecting plasma HDL, Apo-A1 and CETP all of which participate in latter stages of RCT, TRF may also affect cholesterol efflux, an early step in RCT. Furthermore, based on studies by Rajasinghe et al [*], content of these TRF capsules have shown to exhibit anti-cancer effects on NSCLC, further supporting multiple health benefits associated with TRF which was used on our pilot study. To further explain these effects with TRF in ESRD patients, a multi-centered intervention study is being conducted by our lab (clinicaltrials.gov registration number: NCT02358967) with various dialysis units, with much larger number of ESRD population. Therefore, before starting our multi-centered clinical intervention study, we wanted to evaluate the potential effects of TRF capsules that will be given for ESRD patients on cholesterol efflux, and evaluate its anti-cancer effects on NSCLC. To achieve the above, this study was designed to use the contents of new TRF and placebo capsules which are to be used in the clinical trial to evaluate their,

- anti-cancer properties in NSCLC cell line (A549) and
- ability to improve cholesterol efflux in J774A.1 macrophages.

It was hypothesized that contents of TRF capsules can promote cholesterol efflux in J774A.1 macrophages.

## CHAPTER 2: Material and Methods

### 2.1 Cell culture of A549 and treatments.

A549 cells (ATCC) were kindly provided by Prof. Smiti Gupta. Cells were grown in complete media at $37^0$C in 5% $CO_2$ in a humidified atmosphere (standard cell culture conditions for A549 cells). Complete media (RPMI 1640 with 10% FBS and 1% Penicillin/Streptomycin) for A549 cells were prepared by adding 50 ml of FBS (Fisher Scientific, Waltham, MA) and 5 ml of 100 U/ml Penicillin and 100 mg/ml Streptomycin (Fisher Scientific, Waltham, MA) into 500 ml RPMI 1640 media (Fisher Scientific, Waltham, MA). Media was changed every 2 days and cell passaging was done using Trypsin/EDTA (Fisher Scientific, Waltham, MA) when cells became 85-90% confluent. To test the anti-cancer properties, TRF and placebo capsules (Caroteno, Malaysia) manufactured at three different time points, were used. The first batch (TRF 1) had been manufactured in 2012. Batch 2 (TRF 2 and placebo 2) and batch 3 (TRF 3 and placebo 3) were manufactured in 2016 and 2017 respectively. To prepare stock solutions of TRF and placebo, 100 mg of capsule content was taken from each capsule and dissolved in 1 ml of DMSO (Sigma Aldrich, St. Louis, MO) in cryo-vials covered with a foil. Stock solutions were stored in -20°C and used within one month.

### 2.2 Cell viability assays (MTS assay) on A549 cells.

Following procedure was used to test the anti-cancer properties of TRF and placebo for all TRF and placebo batches. Cells were seeded in 96-cell culture plates at a density of $5x10^3$/100µl/well and incubated overnight at standard cell culture conditions for A549

cells. Treatments (20,40,60,80 µg/ml) were prepared by diluting TRF and placebo stock solutions with media. Complete media was used as the control. After 24 hours of seeding, old media was removed, 100 µl of treatment media was added and incubated for another 72 hours at standard cell culture conditions for A549 cells. At the end of 72 hours, Promega Cell titer 96 Aqueous one solution reagent/MTS reagent (Fisher Scientific, Waltham, MA) was diluted with PBS (Fisher Scientific, Waltham, MA) in 1: 1 ratio and 20 µl of diluted MTS reagent was added to each well. Cells were then incubated for another 3 hours at standard cell culture conditions. After 3 hours, plates were shaken for 30 seconds and absorbance was read at 492 nm using SkanIt spectrophotometer (Thermofisher scientific, Waltham, MA). Cell viability was calculated by subtracting post-absorbance from pre-absorbance and relative cell viability was calculated as follows.

$$\text{Relative cell viability} = \frac{\text{Mean cell viability of treatment}}{\text{Mean cell viability of control}} \times 100$$

**2.3 Western-blot analysis for Notch-1 protein.**

To analyze protein expression of Notch-1 gene, A549 cells were seeded at a density of $1x10^6$/ml/petri dish (100 mm) and incubated for 24 hours. Cells were then treated with 40, 80, 120 µg/ml concentrations of TRF for 72 hours. Complete media was used as the control. After 72 hours cells were lysed with a mixture of 1x lysis buffer and 1x protease inhibitor (Cell Signaling Technology, Danvers, MA). Next the protein concentrations were determined using BSA assay (Bio-Rad Laboratories, CA) and 40 µg of total protein was mixed with 4x Lemma buffer (Bio-Rad Laboratories, CA) and loaded into 10% SDS-PAGE gel. After electrophoresis, the gel was transferred into PVDF membrane using

Trans-Blot® TurboTM Transfer System (Holliston, MA). Then the membrane was incubated with 5% Casein for 2 hours at room temperature and incubated with Notch-1 primary antibody (Cell Signaling Technology, Danvers, MA) diluted at 1:4000, for an overnight at 4°C. Then the membrane was washed three times with PBS-T, and incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA), diluted 1:5000, in 2% Casein for 2 hours. Subsequently, the membrane was immersed in the substrate solution (Bio-Rad Laboratories, CA) for 5 minutes and signal intensity was observed using a chemiluminescent imager with chemiDoc XRS (Bio-Rad Laboratories, CA).

**2.4 Cell Culture of J774A.1 and Treatments**

J774A.1 macrophages (ATCC® TIB-67™, Manassas, VA) were grown in complete media at $37^0$C in 10% $CO_2$ in a humidified atmosphere (standard cell culture conditions for J774A.1 cells). Complete media was prepared by adding 50 ml of FBS (Fisher Scientific, Waltham, MA) and 5 ml of 100 U/ml Penicillin and 100 mg/ml Streptomycin (Fisher Scientific, Waltham, MA) to 500 ml of DMEM (Life Technologies (Carlsbad, CA). Cells were first seeded in a T-25 flask and culture media was changed every day until the cells became 85-90% confluent. Once cells reached 85-90% confluency, cells were split using a sterile scraper and seeded in a T-75 flask. Cells were maintained by changing media every day and splitting into 1:3 ratio. Stock solutions from all TRF and placebo batches were prepared as described in section 2.1.

**2.5 Cell viability assay (MTS assay) with TRF and Placebo for J774A.1 cells**

To perform the MTS assay, cells were seeded in 96 cell culture plates at a density of $1x10^4$/100µl/well and incubated overnight at standard cell culture conditions. From TRF

and placebo stock solutions, 20,40, 60, and 80 µg/ml of working treatments were prepared. After 24 hours of seeding, media was removed, 100 µl of treatment media was added and incubated for another 48 hours at standard cell culture conditions. Complete media was used as the control. At the end of 48 hours incubation, MTS assay was performed and relative cell viability was calculated as described in section 2.2.

**2.6 RNA extraction**

For RNA extraction, J774A.1 cells were seeded in sterile cell culture petri dishes (100 mm) at a cell density of $1x10^6$/ml/petridish for 24 hours. After 24 hours, cells were treated with 10 ml of TRF (8 µg/ml, 16 µg/ml) for 24 and 48 hours and with placebo (8 µg/ml, 16 µg/ml) for 48 hours. Complete media was used as the control. After 24 and 48 hours of incubation, RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Hiden, Germany) according to manufacturer's instructions.

Briefly, treatment media was removed and cells were washed with ice-cold PBS. Then 700 µl of QIAzol reagent was added to cell layer and scraped using a sterile cell scraper. Cell homogenate was then incubated at room temperature for 5 minutes and vortexed vigorously for 15 seconds after adding chloroform. The mixture was again incubated at room temperature and centrifuged to obtain the upper aqueous layer containing nucleic acid. To this 100 % ethanol was added, mixed thoroughly and transferred to RNeasy Mini column in a 2 ml collection tube. After centrifugation, the flow through was discarded and the same step was repeated with remaining sample. Next, buffer RWT was added and centrifuged for another 15 seconds. Then, buffer RPE was added to the column and centrifuged for 15 seconds. Same step was repeated for another 2 minutes. To further dry the membrane,

empty column was centrifuged for 1 minute. RNeasy mini column was then placed in an empty 1.5 ml collection tube and 50 µl of RNase-free water was directly added onto the membrane. The column was centrifuged for 1 min at 8000xg to elute bound RNA. Isolated RNA samples were stored at -80°C.

**2.7 RNA quantification and cDNA synthesis**

RNA quantification was performed using Nanodrop. cDNA was synthesized using High Capacity RNA-to-cDNA (Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Briefly, 10 µl of 2x RT buffer, 1 µl of 20x RT enzyme, 2000 ng of extracted RNA and nuclease free water to make the total volume up to 20 µl were mixed in a sterile PCR tube. Reverse transcription reaction was performed by incubating the reaction for 37°C for 60 minutes, stopping the reaction by heating to 95°C for 5 minutes and holding at 4°C. cDNA synthesis was performed in Eppendorf Master cycler realplex 4 (Eppendorf, Hauppauge, NY). cDNA samples were stored in -80°C.

**2.8. Real-time PCR**

Real-time PCR primers for genes to be tested were taken from peer-reviewed papers and are summarized in the Table 2.1 [55-58]. All these primers were purchased from Life Technologies, Carlsbad, CA. Real-time PCR was performed using Power-up SYBR green master mix (Fisher Scientific, Waltham, MA) in Eppendorf Master cycler realplex 4 (Eppendorf, Hauppauge, NY). GAPDH was used as the house-keeping gene. Cycle parameters used were as follows: UDG activation at 50°C for 2 minutes, extension by dual lock DNA polymerase at 95°C for 2 minutes, denaturation at 95°C for 15 seconds, annealing at 55-60 °C for 15 seconds and extension at 72°C for 1 minute. Gene expression

was expressed as fold change ($2^{-\Delta\Delta CT}$). Two-fold increase (≥2) or 2-fold decrease (≤ 0.5) compared to the control in each treatment group was considered to be significant.

**Table 2.1 Real-time PCR primers**

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| GAPDH [57] | 5-AACTTTGGCATTGTGGAAGGG- 3 | 5-GACACATTGGGGGTAGGAACAC-3 |
| PPAR-γ [56] | 5-GGAAGACCACTCGCATTCCTT-3 | 5-GTAATCAGCAACCATTGGGTCA-3 |
| CD-36 [55] | 5-AGGTCTATCTACGCTGTGTTC-3 | 5-TGGTTGTCTGGATTCTGGAG-3 |
| SRA1 [55] | 5-AGAGGGCTTACTGGACAAACTG-3 | 5-GGCTTTCCTGGTGCTCCTG-3 |
| SRB1 [57] | 5-ATGGGCCAGCGTGCTTTTATGA-3 | 5-AACCACAGCAACGGCAGAACTA-3 |
| LXR-α [59] | 5-CAATGCCTGATGTTTCTCC-3 | 5-TGACTCCAACCCTATCCCTA-3 |
| ABCG1 [58] | 5-TTCATCGTCCTGGGCATCTT-3 | 5-CGGATTTTGTATCTGAGGACGAA-3 |
| ABCA1 [57] | 5-AGCTGCCCCATCATGTAAAG-3 | 5-GGGAGAAGAGCGTGCTAATG-3 |

### 2.9 Statistical Analysis

Statistical analysis between treatment and control groups were analyzed using one-way ANOVA (ezANOVA, USA). Statistical significance was set at $p<0.05$.

# CHAPTER 3: Results

## 3.1 TRF dose dependently inhibited A549 cell proliferation.

TRF is known to inhibit cancer cell proliferation. To verify that contents of TRF capsules are active, the anti-cancer properties of different TRF batches (TRF 1, 2 and 3) were tested using NSCLC cell line (A549). A549 cells were seeded at a density of $5x10^3$ cells/100µl/well and treated with 40, 80, 120 µg/ml of TRF or placebo for 72 hours. Complete media was used as the control. Based on the results of MTS assays, all TRF batches significantly inhibited growth of A549 cells at the concentration of 80 µg/ml and no significant inhibition was observed with placebo treatments (**Figure 3.1 (A)**).

### 3.1.1 TRF dose dependently inhibited Notch-1 protein expression

Expression of Notch-1 is known to modulate cancer cell proliferation in many types of cancers such as colon, pancreatic, lung, breast and skin. To test the effects of TRF on notch-1 expression in A549 cell lines a western-blot analysis was performed. Cells were seeded at a density of $1x10^6$ cells/ml and treated with 40,80,120 µg/ml of TRF for 72 hours. Based on western blot data, 40, 80, 120 µg/ml of TRF dose dependently decreased Notch-1 protein expression **(Figure 3.1(B))**.

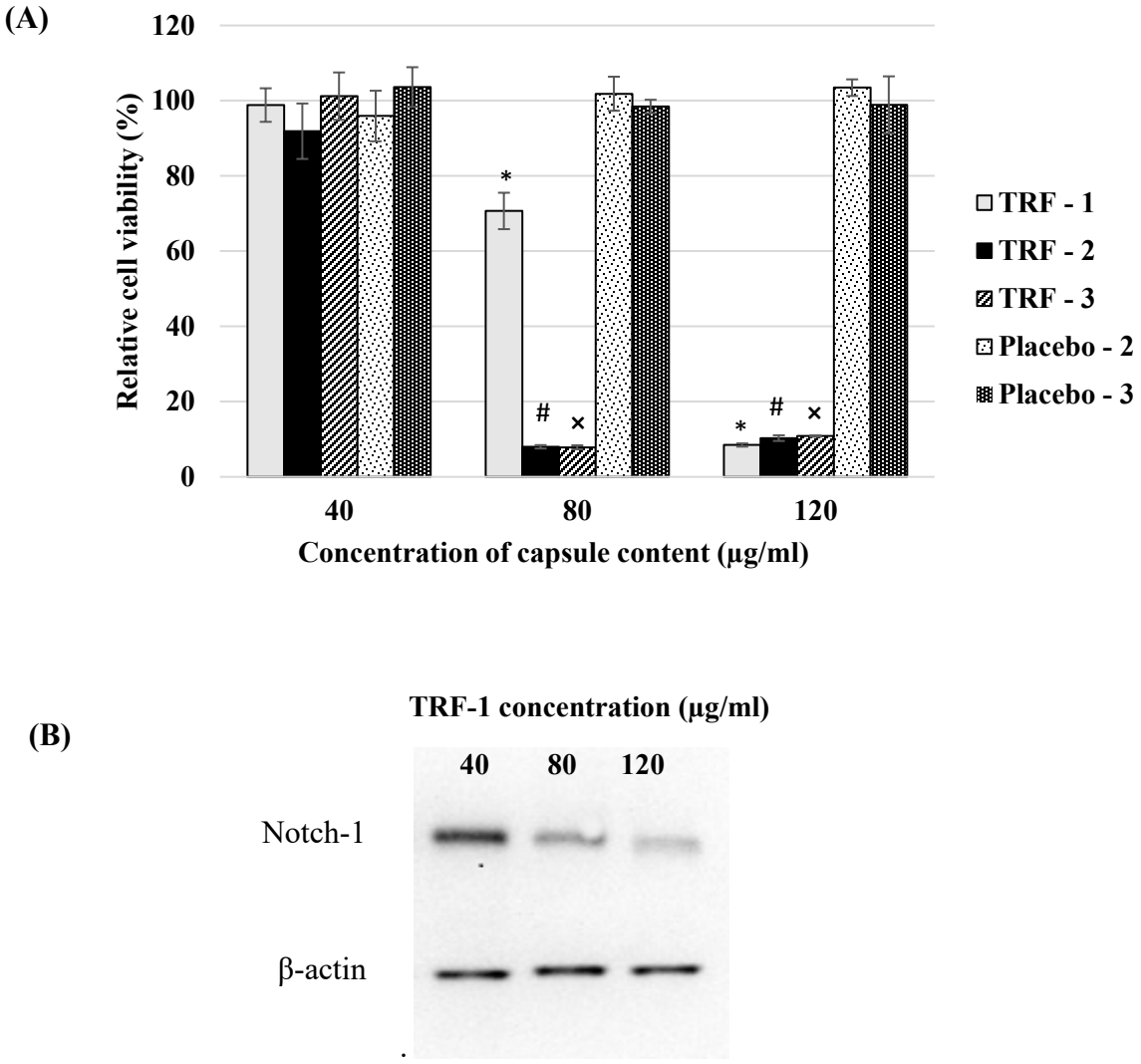



**Figure 3.1 Effects of TRF and placebo batches on A549 cell proliferation and effect of TRF on Notch-1 expression**

**(A)** MTS assay was used to determine anti-cancer effects of different batches of TRF and placebo on A549 cells. Cells were seeded at a density of 5000 cells/100µl/well and incubated with 40, 80 and 120 µg/ml of TRF and placebo separately for 72 hours. Vertical axis shows the mean relative cell viability ± SD. Statistical significance determined using One way ANOVA with TukeyHSD multiple comparison test (ezANOVA, USA). Statistical significance was set at $p < 0.05$. Statistical significance for TRF 1 compared to control is denoted as "*". Statistical significance for TRF 2 compared to control is denoted as "#". Statistical significance for TRF 3 compared to control is denoted as "×".
**(B)** Expression of TRF on Notch-1 protein expression was analyzed using western-blot analysis, after treating cells ($1x10^6$/ml) with 40, 80, 120 µg/ml of TRF for 72 hours.

**3.2 TRF-2 concentrations up to 20 μg/ml did not cause cytotoxicity in J774A.1 cells.**

To determine the non-cytotoxic concentrations of TRF for J774A.1 cells, MTS assay was performed. Cells were seeded with $1x10^4$ cells/100 μl/well and treated with 20, 40, 60, 80, 100, 120 μg/ml concentrations for 48 hours. Based on the results of MTS assay more than 90% relative cell viability was observed with 20 μg/ml of TRF (**Figure 3.2**). Concentrations of 40, 60,80, 100 and 120 μg/ml of TRF, significantly reduced cell growth with relative cell viabilities of 72%, 31%, 3%, 2.3% and 2.1% respectively, compared to the control. No significant effect was observed with treatment with placebo on cell growth at given concentrations for 48 hours. Based on MTS assay data, concentrations less than 20 μg/ml were selected for gene expression experiments.

**3.3 TRF-2 increased PPAR-γ gene expression.**

Increased expression of PPAR-γ is known to increase cholesterol efflux. To test the effect of TRF and placebo on PPAR-γ gene expression**,** cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 μg/ml of TRF-2 for 24 hour and 48 hours and with placebo (8, 16 μg/ml) for 48 hours. Complete media was used as the control. PCR data revealed that treatment with TRF-2 for 24 hours with 8 and 16 μg/ml caused a significant dose dependent increase in PPAR-γ compared to the control (**Figure 3.3 (A))**. In 48 hour treatments, no significant change was observed at 8 μg/ml, but a significant 2.93 increase in fold change was observed for 16 μg/ml compared control (**Figure 3.3 (B))**. Treatment with placebo for 48 hours did not cause any dose dependent effect compared to control

(**Figure 3.3 (C)).** Among tested conditions, highest increase of PPAR-γ (2.93x increase compared to control) was observed with 16 µg/ml for 48 hours.

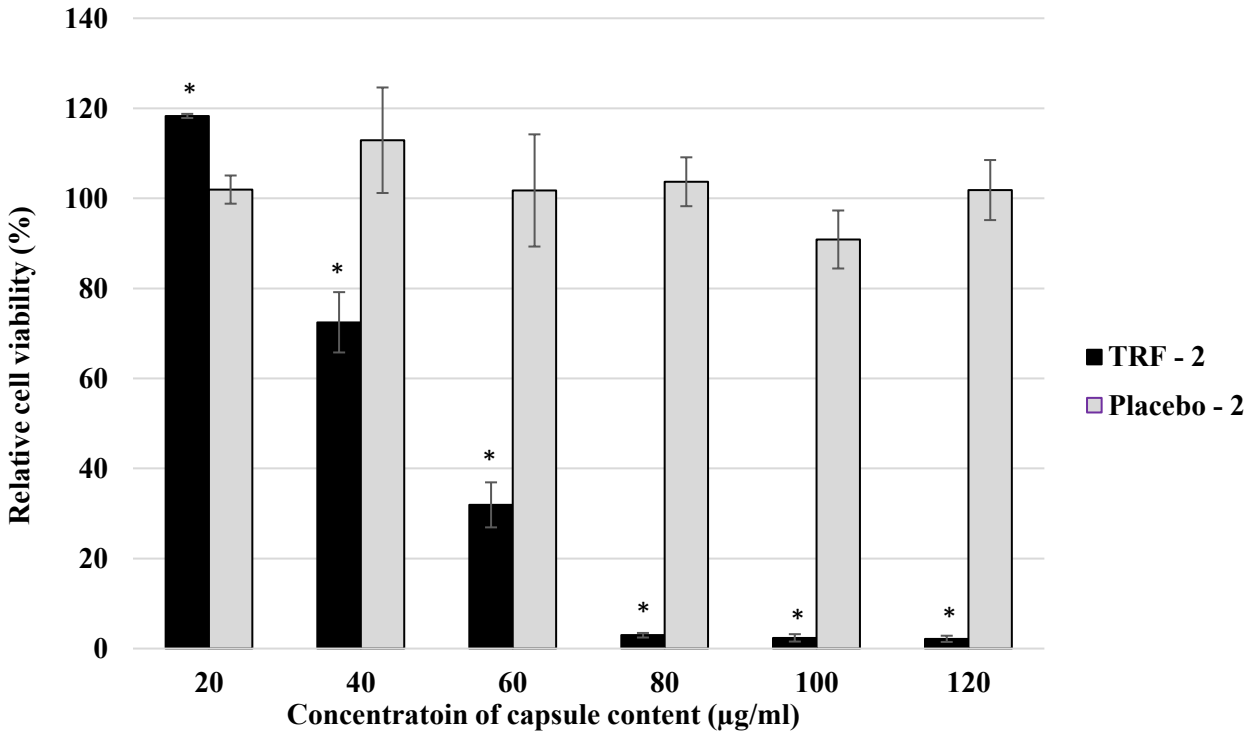



**Figure 3.2 Effects of TRF-2 and placebo-2 on cell viability of J774A.1 macrophages**

MTS assay was used to determine cytotoxic effects of TRF-2 and placebo-2 on J774A.1 macrophages. Cells were seeded at a density of $1x10^4$/100µl/well and incubated with 20,40, 60, 80, 100 and 120 µg/ml of TRF-2 and placebo-2 separately. Complete media was used as the control. Vertical axis shows the mean relative cell viability ± SD. Statistical significance determined using One way ANOVA with TukeyHSD multiple comparison test (ezANOVA, USA). Statistical significance compared to control is denoted as "*" ($p < 0.05$).

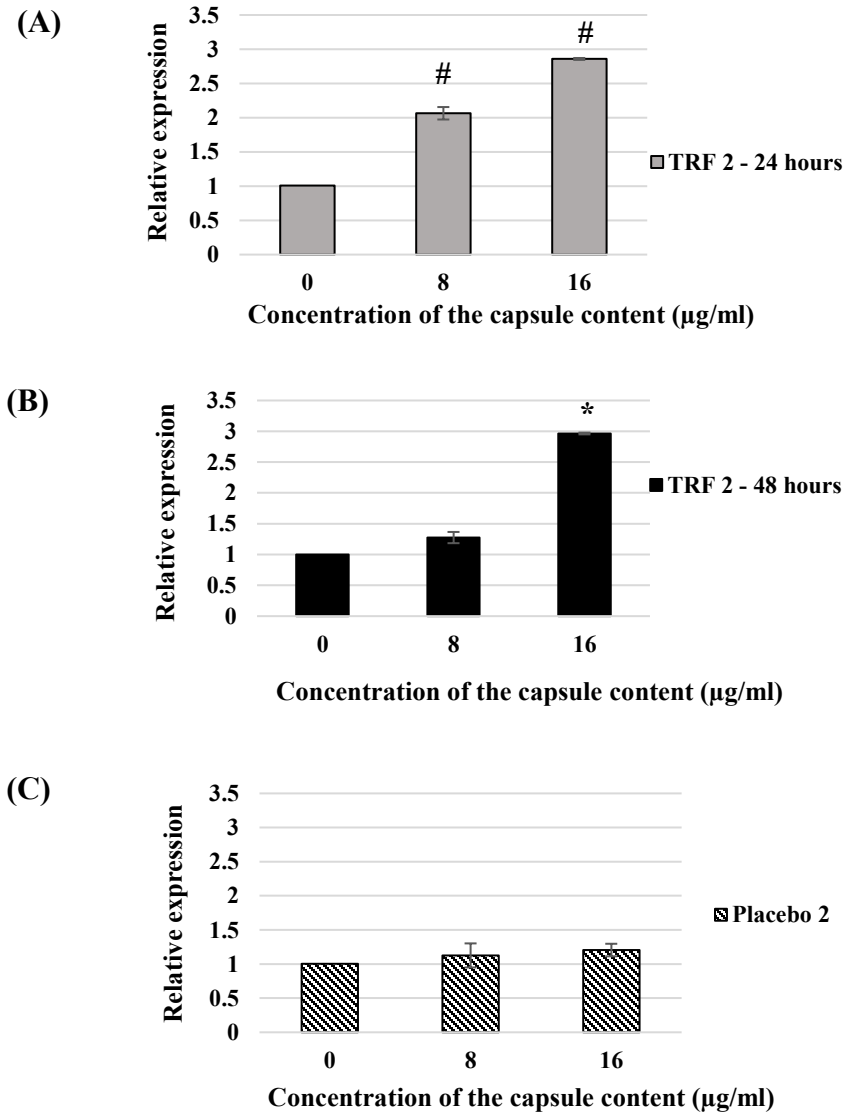



**Figure 3.3 Effects of TRF-2 and placebo-2 on PPAR-γ**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene.

**3.4 TRF-2 decreases SRA1 gene expression.**

SRA1 facilitates uptake of oxidized LDL into the macrophages. To test the effect of TRF-2 on expression SRA1 expression**,** cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 µg/ml of TRF-2 for 24 hour and 48 hours and with placebo-2 (8, 16 µg/ml) for 48 hours. Based on real-time PCR data, treatment with 8 and 16 µg/ml of TRF-2 at 24 hours did not show any significant changes in the expression of SRA1 compared to the control. (**Figure 3.4 (A))**. With 48-hour treatment, no significant change was observed for 8 µg/ml of TRF-2 compared to control (**Figure 3.4 (B))**. However, a significant reduction was observed with 48-hour treatment at 16 µg/ml compared to the control. As shown in **Figure 3.4 (C)**, no dose dependent and/or time dependent effect was observed with placebo. Among tested conditions, lowest expression of SRA1 (4.9x decrease compared to control) was observed with 16 µg/ml for 48 hours.

**3.5 TRF-2 increased CD-36 gene expression.**

CD-36 is also involved in the uptake of oxidized LDL into macrophages. To test the effect of TRF-2 gene expression CD-36**,** cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 µg/ml of TRF-2 for 24 hour and 48 hours and with placebo (8, 16 µg/ml) for 48 hours. Treatment with TRF-2 for 24 hours with 8 and 16 µg/ml did not cause any significant changes in the expression of CD-36 compared to control (**Figure 3.5 (A)**).

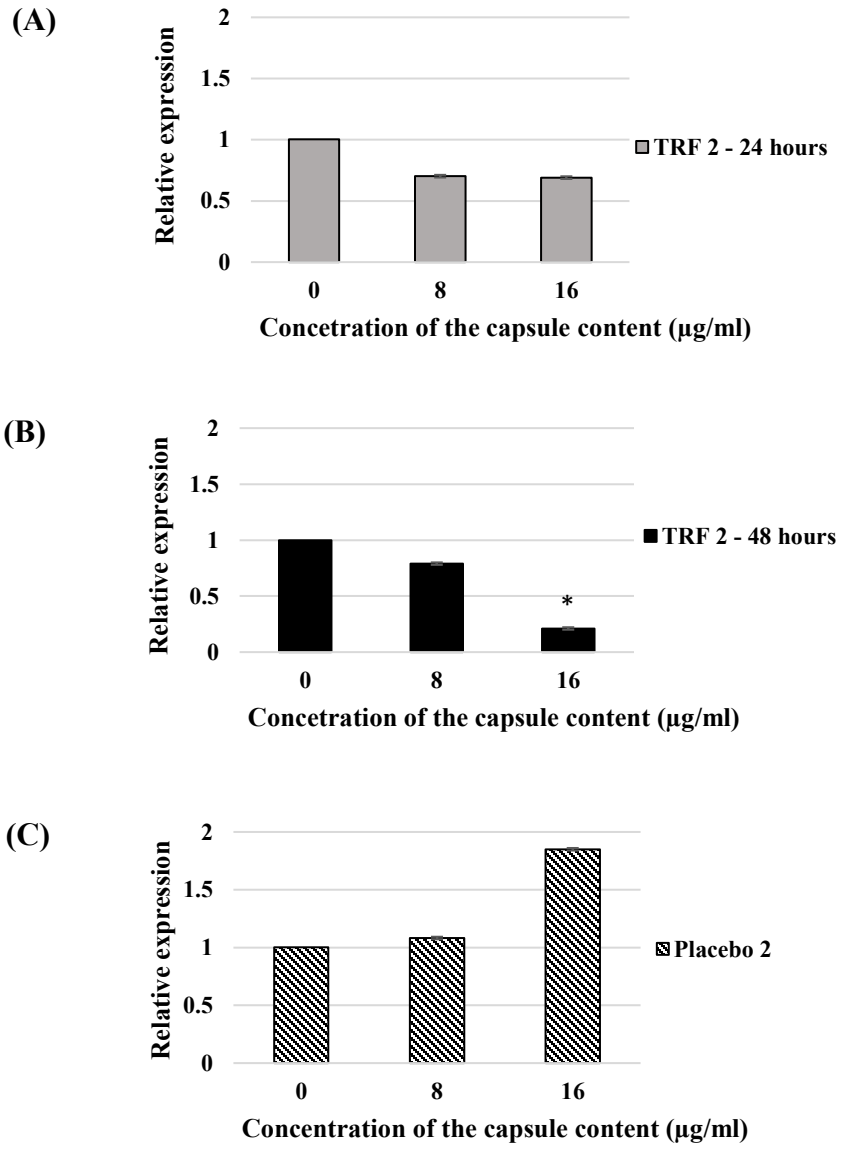



**Figure 3.4 Effects of TRF-2 and placebo-2 on SRA1**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene.

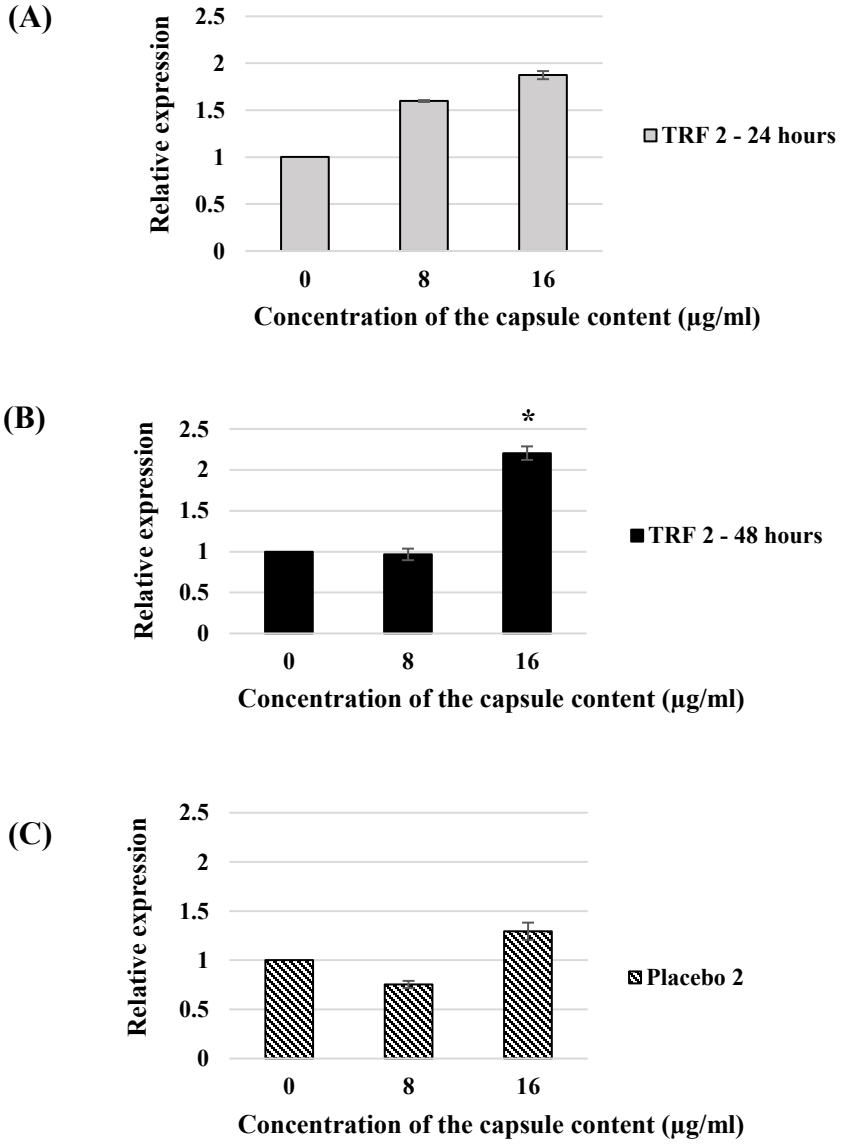



**Figure 3.5 Effects of TRF-2 and placebo-2 on CD-36**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene.

No significant changes were observed with 48-hour treatment at 8 μg/ml but a significant increase in CD 36 was observed at 16 μg/ml compared to the control (**Figure 3.5 (B)**). No significant changes were observed with 8 and 16 μg/ml of placebo compared to control for 48 hours (**Figure 3.5 (C)**). Based on these data, highest significant increase of CD-36 expression (2.2x compared to control) was observed with 16 μg/ml TRF-2 for 48 hours, under tested conditions.

**3.6 TRF-2 increased SRB1 gene expression.**

SRB1 facilitates cholesterol efflux by releasing free cholesterol into HDL-2 particles. Therefore, effect of TRF-2 and placebo-2 on SRB1 gene expression was tested using real-time PCR. Cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 μg/ml of TRF-2 for 24 hour and 48 hours. Cells were also treated with placebo-2 (8, 16 μg/ml) which contained wheat germ oil as the major component, for 48 hours. Based on real-time PCR data, treatment with 8 and 16 μg/ml of TRF-2 for 24 hours did not cause any significant changes compared to control (**Figure 3.6 (A)**). In contrast, 48-hour treatment caused a significant dose dependent increase in SRB1 expression compared to control (**Figure 3.6 (B)**). Treatment with 8 and 16 μg/ml of placebo-2 did not cause any significant changes in SRB1 expression after 48 hours treatment (**Figure 3.6 (C)**). Based on these results, highest significant increase SRB1 expression occurred with TRF-2 of 16 μg/ml for 48 hours (4x increase compared to control) under tested conditions.

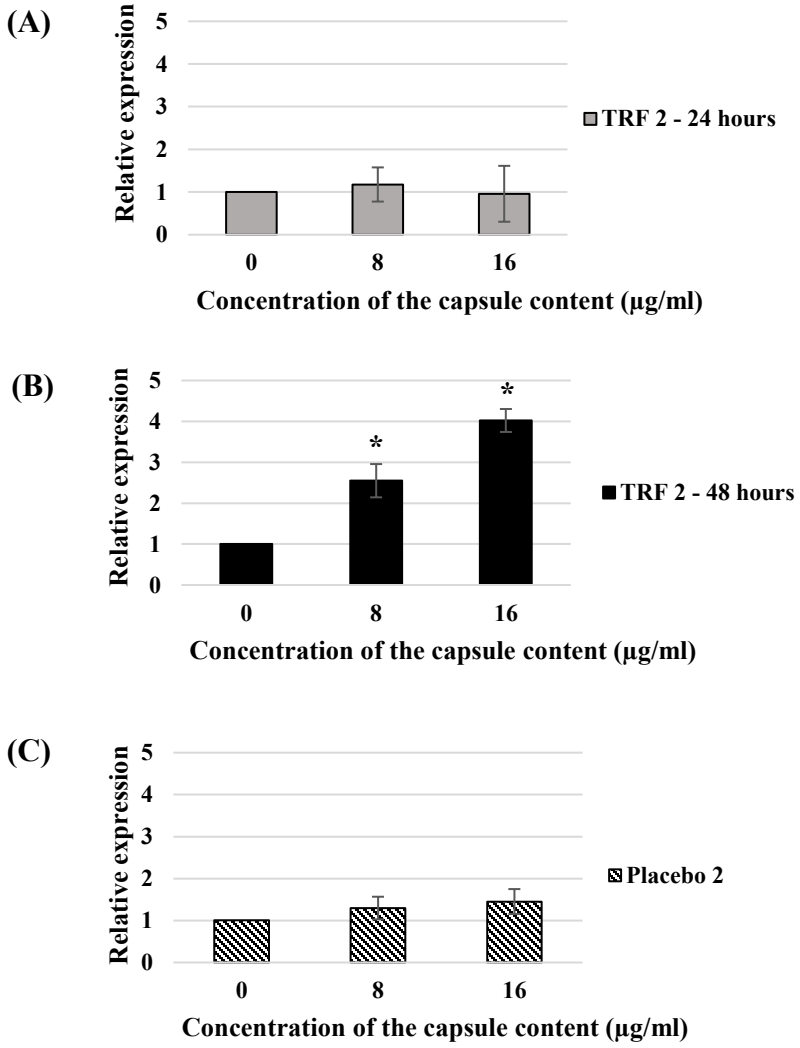



**Figure 3.6 Effects of TRF-2 and placebo-2 on SRB1**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene.

**3.7 TRF-2 did not affect LXR-α gene expression.**

LXR-α is a downstream gene of PPAR-γ and is an upstream gene to cholesterol efflux genes such as ABCA1 and ABCG1. To test the effect of TRF on LXR-α and ABCG1 gene expressions**,** cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 μg/ml of TRF-2 for 24 hour and 48 hours and with placebo (8, 16 μg/ml) for 48 hours. Based on PCR data, no significant effects were observed with 8 and 16 μg/ml of TRF-2 at 24 hours and 48 hours (**Figure 3.7 (A and B))**. Also, no significant effects were observed with treatment of 8 and 16 μg/ml of placebo-2 for 48 hours (**Figure 3.7 (C))**.

**3.8 TRF-2 did not affect ABCG1 gene expression**.

ABCG1 is a cholesterol efflux gene downstream to LXR-α. No significant changes were observed with ABCG1 expression under tested conditions. (**Figures 3.8 (A), (B) and (C)**).

**3.9 TRF-2 decreased ABCA1 gene expression.**

ABCA1 aids efflux of free cholesterol into Apo-A1 particles. To test the effect of TRF-2 and placebo-2 on LXR-α and ABCG1 gene expressions**,** cells were seeded at a density of $1x10^6$ cells/ml and treated with 8, 16 μg/ml of TRF-2 for 24 hour and 48 hours and with placebo-2 (8, 16 μg/ml) for 48 hours. **Figure 3.9 (A and B)** summarized effects of TRF-2 on ABCA1 overtime. Treatment with 8 and 16 μg/ml for 24 hours significantly reduced ABCA1 expression compared to the control. A significant 2.8 reduction was observed for both 8 and 16 μg/ml with 48 hours treatment compared to control. Treatment with placebo-2 did not cause any significant changes in ABCA1 gene expression **(Figure 3.9 (C))**.

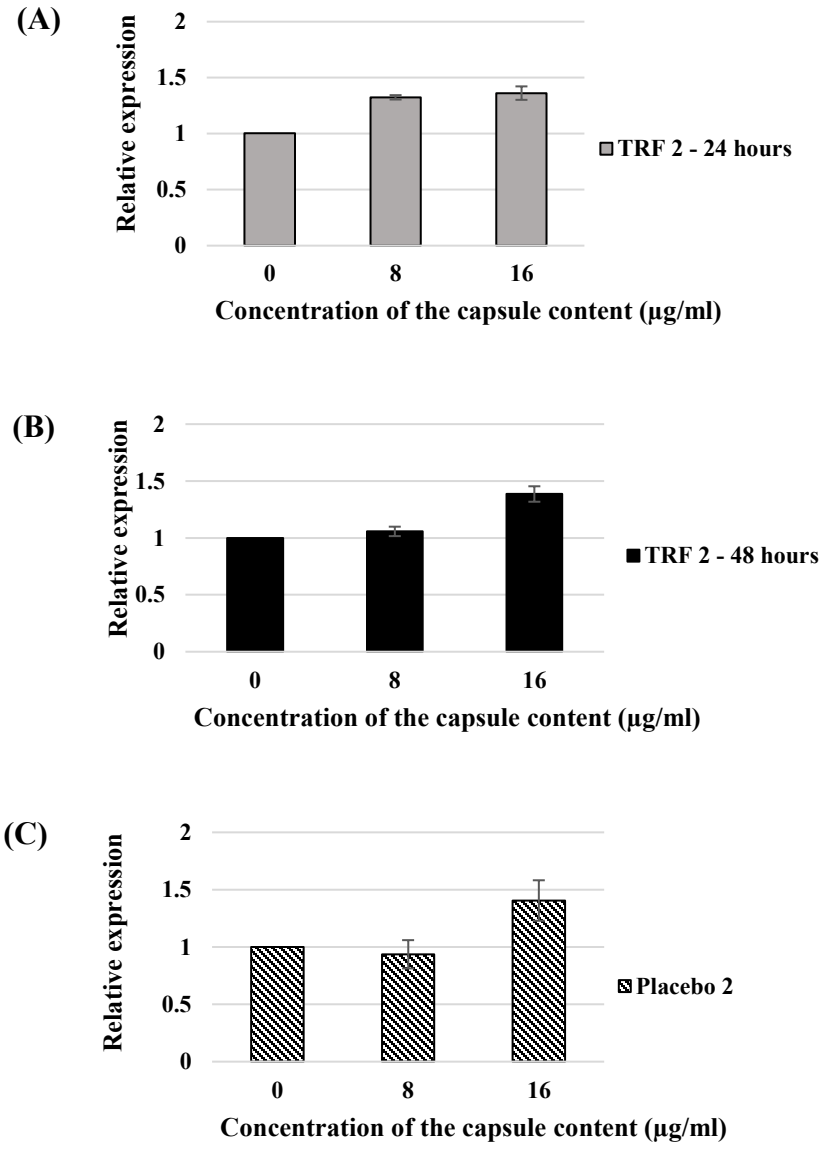



**Figure 3.7 Effects of TRF-2 and placebo-2 on LXR-α**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene

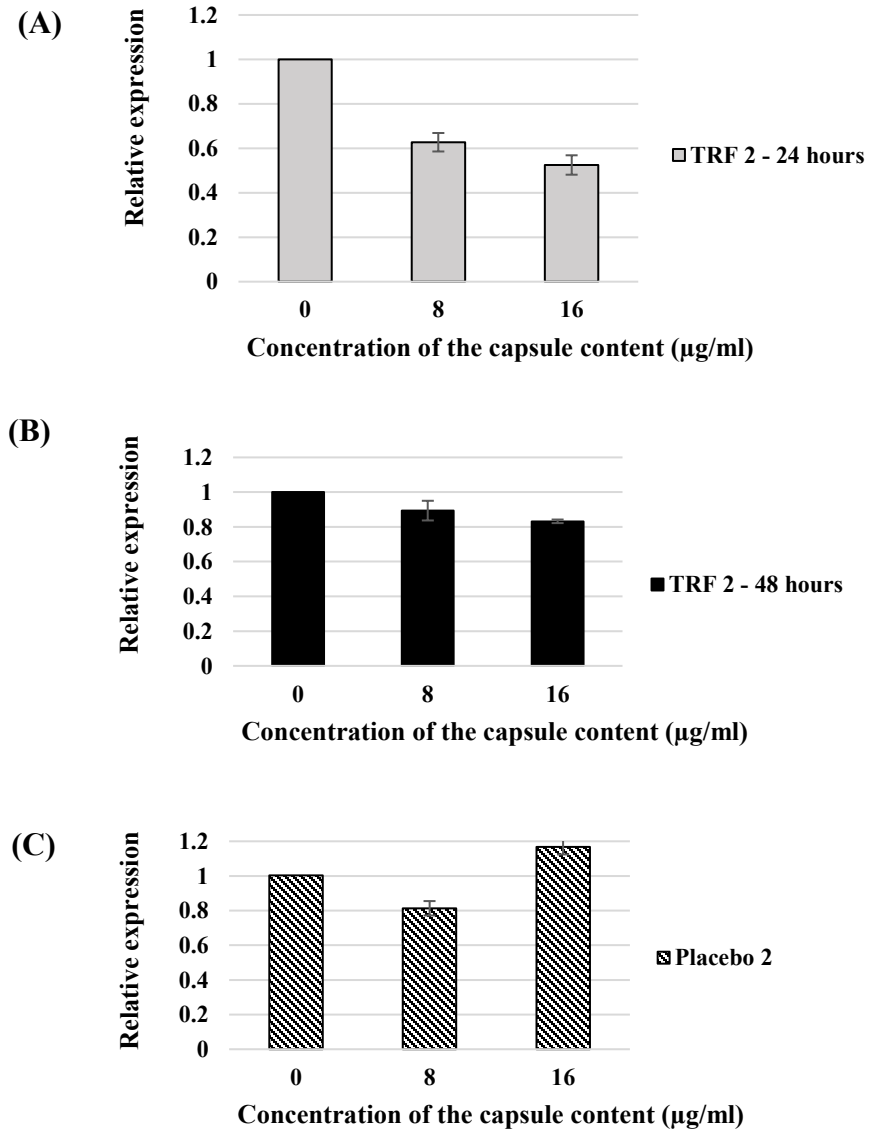



**Figure 3.8 Effects of TRF-2 and placebo-2 on ABCG1**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene.

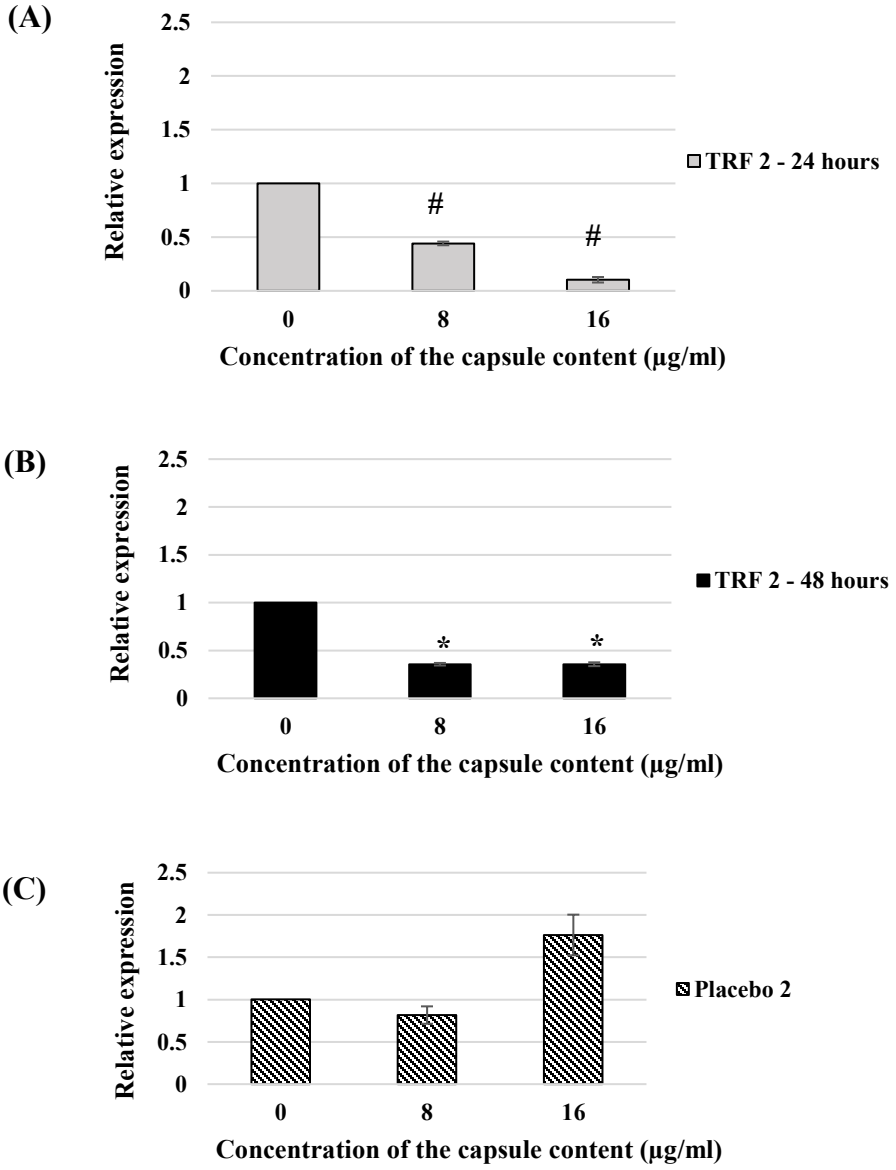



**Figure 3.9 Effects of TRF-2 and placebo-2 on ABCA1**

Effects of TRF-2 and placebo-2 on PPAR-γ was tested using real-time PCR. J774A1 cells were seeded at a cell density of $1x10^6$/ml for 24 hours. Cells were then treated with 8 µg/ml 16 µg/ml of TRF-2 for 24 hours and 48 hours and with placebo-2 (8 µg/ml 16 µg/ml) for 48 hours. **(A)** Effects of TRF-2 at 24 hours **(B)** Effect of TRF-2 at 48 hours **(C)** Effects of placebo at 48 hours. The vertical axis indicates the mean relative expression (calculated using $2^{-\Delta\Delta CT}$ method) ± SD. Two-fold increase or decrease compared to control was considered significant. Significant difference in TRF 2- 24 hours compared to control is denoted as "#". Significant difference in TRF 2- 48 hours compared to control is denoted as "*". GAPDH was used as the house keeping gene

**Discussion:**

This study was conducted as a preliminary step of predicting potential beneficial effects of TRF capsules that will be given to ESRD patients in our multi-centered clinical intervention study. Based on our pilot study it was found out that TRF (78.7% tocotrienols, 21.3% tocopherols) increased plasma HDL, Apo-A1 and decreased CETP activity in ESRD patients [49]. In addition, using same TRF capsules, Rajasinghe et al found out TRF inhibit NSCLC cancer cell (A549 cells) proliferation via inhibiting Notch-1 pathway, supporting that TRF used in our pilot study not only can improve lipid profiles but also have anti-cancer properties.

TRF-1 (78.7% tocotrienols, 21.3% tocopherols) was manufactured in 2012 and the new TRF (TRF-2 and TRF-3) were manufactured in late 2016 and early 2017 respectively. TRF 2 and 3 have a similar composition (76.53% tocotrienols and 23.47% tocopherol) to the capsules that will be given to ESRD patients in our multi-centered study and were purchased as samples. Since Rajasinghe et al have shown anti-cancer effects of TRF on A549 cells, a NSCLC cell line*, we used the same cell line to identify whether the contents of new TRF capsules are active, using well-established anti-cancer properties of palm oil derived TRF on NSCLC cell line, A549. Based on the MTS assay, we found out that all three TRF batches significantly inhibited cancer cell proliferation at 80 μg/ml compared to control used for each batch. No significant cell growth inhibition was observed with placebo. Despite of having similar composition to TRF 1, TRF 2 and 3 showed more potency in cell growth inhibition at 80 μg/ml. Degradation of TRF in TRF-1 over time can

be a possible reason to the reduced cell growth seen with TRF-1 compared to TRF 2 and 3 which were manufactured recently.

Dose dependent decrease in cancer cell proliferation due to TRF -1 was further confirmed by a western-blot analysis of Notch-1 gene, which plays a critical role in cancer cell proliferation, differentiation and apoptosis in NSCLC. Effects of TRF on Notch-1 expression in NSCL has been extensively studied by Gupta lab in our department [50-52]. Ji et al found that treatment with pure δ-tocotrienol isomer, dose dependently inhibits proliferation of human NSCLC cell lines, A549 and H1299. Similarly, treatment with pure δ-tocotrienol isomer dose dependently reduced Notch-1 mRNA and protein expression [50, 52]. Studies by Rajasinghe et al[*] also reported similar effects on cancer cell proliferation and Notch-1 expression with TRF extracted from TRF capsules, further confirming beneficial effects of TRF on NSCL. Based on the results of MTS assays it was identified that contents in new TRF (TRF-2, TRF-3) capsules are active.

Since cholesterol efflux can be promoted due to net effect of cholesterol influx and efflux gene expression, we then tested the effects of TRF-2 on expression of several genes in cholesterol efflux. Cholesterol efflux is the first step in reverse cholesterol transport, which is now considered as a novel predictor of CVD risk. To study the effects of TRF-2 on cholesterol efflux , J774A.1 cells, a well-established cell culture model which is widely used for cholesterol efflux studies was used [15]. Based on a review by Annema et al, 30 out 60 reported RCT studies are conducted with J774A.1 cell line, whereas out of those

[*] Rajasinghe, L. and S. Gupta, *Tocotrienols suppress non-small lung cancer cells via downregulation of the Notch-1 signaling pathway (644.1).* The FASEB Journal, 2014. **28**(1 Supplement): p. 644.1

30, 10 reported effect of different commercially available drugs on RCT [15]. Furthermore, it has also been used to test the effect of different naturally occurring bioactive compounds such as resveratrol, α-lipoic acid, curcumin on cholesterol efflux [29, 30, 32].

The non-cytotoxic treatment concentrations of TRF-2 for J774A.1 cell line was determined using MTS assay and based on MTS assay results, no cytotoxic effects were observed with concentrations from 0-20 μg/ml. Therefore, 8 and 16 μg/ml were selected as treatment concentrations. These treatment concentrations are in the range of treatment concentrations which have been used in several TRF studies [48, 60, 61]. In a study performed to test anti-inflammatory properties of palm oil TRF on LPS- induced mouse peritoneal macrophages, TRF concentrations of 5, 10 and 30 μg/ml significantly reduced expression of inflammatory markers such as nitric oxide (NO), prostaglandin E2 ($PGE_2$), and proinflammatory cytokine (TNF-α, IFN-γ, IL-1β and IL-6) production [60].

In another study which tested the potential effects of palm oil TRF on inhibition of vascular adhesion molecules in endothelial cells, treatment with lower TRF concentrations ranging from 10-50 ng/ml, did not affect cell viability and at the same time caused a significant dose dependent reduction of adhesion molecules expression [61]. However, concentrations above 20 μg/ml, caused a significant cell death in this study [61]. Furthermore, treatment with 0.5, 1.0, 5.0 μg/ml of palm oil TRF has shown dose dependent anti-inflammatory effects on THP-1 macrophages [48]. These studies further validate results obtained from MTS assay supporting that palm oil TRF can be effective at very low concentrations.

PPAR-γ is an important gene which is known to regulate anti-inflammatory and anti-atherosclerosis properties. In this study, we found out that treatment with TRF-2 significantly increased PPAR-γ expression. Similar agonistic effects of TRF on PPAR-γ is reported in a study conducted by Li et using both macrophage and atherosclerotic mouse model [54]. More importantly TRF used in this study has a similar origin and composition of (76.53% tocotrienols and 23.47% tocopherol) to TRF-2 used in our study (74.68% tocotrienols and 23.5% tocopherol). In the cell culture model, treatment with 0.625, 2.5, 10, 40 μg/ml of palm oil TRF dose dependently increased PPAR-γ in RAW264.7 murine macrophages [54]. In fact, the increase of PPAR-γ observed at 10 μg/ml was similar to that of 1 μM rosiglitazone, a synthetic PPAR-γ agonist. In the mouse model, supplementation of ApoE−/− mice with an atherogenic diet together with 0.05% TRF, or 0.2% TRF for 16 weeks significantly reduced the average lesion/aorta area to 35.5% and 28.4% respectively[54]. These data further support our findings that TRF can act as a possible PPAR-γ agonist.

PPAR-γ is known to modulate cholesterol influx by altering expression of its downstream cholesterol influx genes such as SRA1 and CD-36. Increased PPAR-γ expression is reported to increase CD36 while reducing SRA1, leading no net effect on cholesterol influx [20, 24, 25, 62]. In our study, we found out that treatment with TRF-2 significantly decrease SRA1 and significantly increase CD-36 expression. In a study performed by Moore et al, Troglitazone which is a synthetic PPAR-γ agonist and an antidiabetic thiazolidinediones (TZDs) drug, increased the expression of CD 36 and while decreasing SRA1, leading no net cholesterol influx in murine macrophages [24]. Rosiglitazone which is also a TZDs

drug and a synthetic PPAR-γ agonist, has also shown similar effects [25]. These data further support potential PPAR-γ agonistic properties of TRF observed in this study.

SBR1 is a cholesterol efflux genes, which is downstream to PPAR-γ. Upregulation of PPAR-γ is known to increase SRB1 expression. Based on our results, TRF significantly increased SRB1 expression. Several studies report similar effects on SRB1 using PPAR-γ agonists. In a study conducted by Chinetti et al, C57BL/6J ApoE−/− mice supplemented with standard chow diet (control group) or chow diet containing 0.2% (wt/wt) fenofibrate (synthetic PPAR-γ. agonist) or 1.4% (wt/wt) troglitazone ( synthetic PPAR-γ agonist) for two weeks, an increased SRB1 protein expression was observed [62]. In another study, treatment of THP-1 macrophages for 24 hours with another synthetic PPAR-γ agonist, Telmisartan (1-20 μM) dose dependently increased SRB1 protein expression [63].

In addition to these synthetic compounds, ferulic and caffeic acid which are naturally found in coffee has shown to increase SRB1 gene expression, dose dependently (0.25 – 1 μM) in THP-1 macrophages when treated for 6 hours[33]. These studies further support that TRF can increase SRB1 gene expression, via exerting PPAR-γ agonistic properties.

Although the effect of SRB1 gene expression on SRB1-mediated cholesterol efflux is not measured in our study, results from recent studies show that increased SRB1 expression promotes SRB1 mediated cholesterol efflux. Ji et al reported that overexpression of SRB1 in Chinese hamster ovary (CHO) cells, caused 4 fold increase in cholesterol efflux compared to control CHO cells [64]. Similarly, overexpression of SRB1 caused a 2.2 fold increased in cholesterol efflux to HDL, in RAW264.7 murine macrophages [65]. Since

TRF used in this project also increased SRB1 expression, it can be postulated that TRF used in this study may increase SRB1 mediated cholesterol efflux.

PPAR-γ-LXR-α- ABCA1/ABCG1 pathway is one of the most studied cholesterol efflux pathway. Upregulation of PPAR-γ is known in increase gene expression of LXR-α-ABCA1 and ABCG1 genes. Although TRF increased PPAR-γ expression, we did not observe an increase in LXR-α and ABCG1 expression.

In the study by Li et al, although treatment with TRF (0.625 – 40 μg/ml) significantly increased PPAR-γ expression, it did not cause a significant LXR-α expression, compared to 1 μM of synthetic LXR agonist T0901317 in RAW 264.7 macrophages [45]. However, in their in vivo study, Apo E−/− mice fed with an atherogenic diet together with 0.05% TRF or 0.2% TRF for 16 weeks, caused a significant increase in LXR-α, ABCG1 and ABCA1 expression compared to synthetic PPAR-γ agonist, rosiglitazone [54]. In addition, in our study we observed that TRF-2 treatment significantly reduced ABCA1 gene expression. Therefore, to get more conclusive results on effects of TRF on PPAR-γ-LXR-α- ABCA1/ABCG1 pathway, further studies are warranted.

Effects of TRF on PPAR-γ, SRA1, CD 36, LXR-α, ABCG1 and ABCA1 obtained in our study are summarized in the **Figure 4.1**.

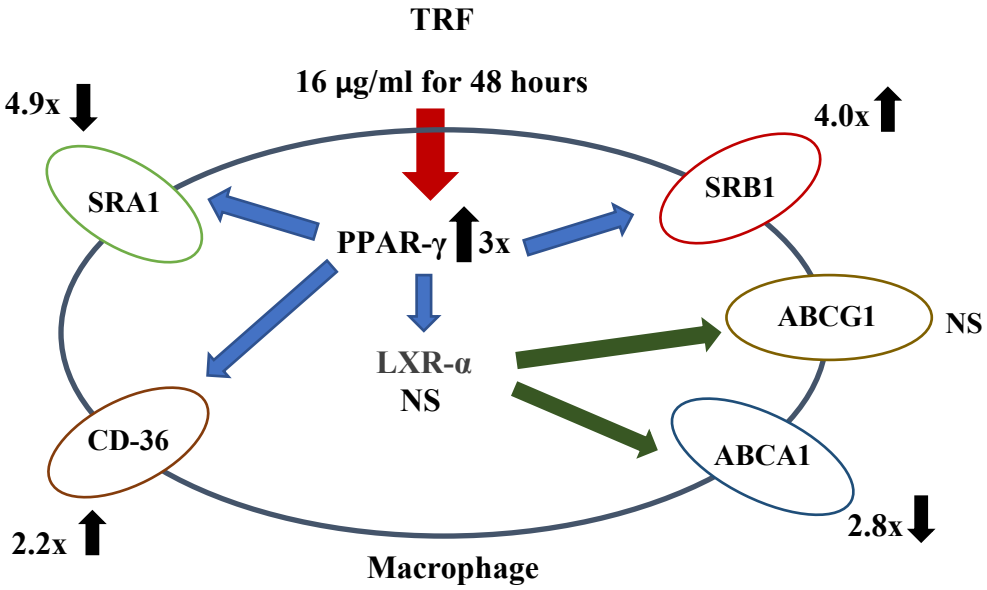



**Figure 4.1: Summary of effects observed with TRF (16 µg/ml for 48 hours) PPAR-γ and its downstream efflux and influx genes in J774A.1 macrophages**

TRF and placebo treatments used in this study were prepared from commercially available capsules and both contained wheat germ oil. In contrast to placebo, TRF contained 74.68% tocotrienols and 23.5% tocopherol per capsule (61.01 mg of tocopherols and 198.56 mg of tocotrienols; total amount 260.17 mg). In fact, effects of TRF on these genes were observed with ~1.6x $10^4$ diluted solution (16 µg/ml) of original TRF-2 capsule (260.17 mg/ml). More importantly, significant changes of gene expression observed in the study are seen only with TRF treatment and placebo treatment did not cause any significant changes. Therefore, the results obtained from the study can be attributed to effects of TRF.

This is further supported by the pre-clinical study conducted by our lab [49]. In this study hemodialysis patients were supplied with 2 capsules of TRF-1 or placebo, 3 times a week for 16 weeks. TRF group caused a significant increase in plasma HDL at both $12^{th}$ week and $16^{th}$ week compared to placebo group. Similarly, TRF treatment increased plasma Apo A1 levels and decreased plasma TG level compared to placebo at week 12. Furthermore,

TRF treatment increased CETP activity in both 12$^{th}$ and 16$^{th}$ week compared to placebo indicating potential beneficial effects of TRF.

However, TRF used in this study (TRF-2) contained 23.5 % of α-tocopherol in addition to 74.68% of tocotrienols. Several research articles suggest that tocotrienols have superior activity over tocopherol. In the study by Li et al, where significant increase in PPAR-γ in THP-1macrophages was observed with 24 hour treatment, 25 μM of α-tocopherol did not caused significant change in PPAR-γ and LXR-α [54]. Furthermore, superior action of TRF over tocopherols is reported in related to anti-oxidant [40, 41, 66-68], anti-inflammatory [69], anti-cancer [70-72], hypocholesterolemic [73, 74] and neuroprotective [75] properties. These finding suggest that, PPAR-γ agonistic effects observed in our study can be attributed to tocotrienols present in TRF. However, to conclude this, further studies are warranted with pure tocotrienols and tocopherol isomers.

Based on the results of cholesterol efflux gene expression study, it can be postulated that TRF may have potential PPAR-γ agonistic effects, that can increase net cholesterol efflux. However, to further conclude the gene expression data, further experiments that evaluate time and dose dependent effects of TRF on protein expression of these receptors are warranted. More importantly, a cholesterol efflux assay has to be conducted to understand the overall effect of TRF on cholesterol efflux. In addition, selectively over expressing and inhibiting each influx and efflux gene in-vitro and assessing effects of TRF on their gene expression, protein expression and their contribution to cholesterol efflux may further help understand the targeted effect of TRF on the unique role played by each protein.

## CHAPTER 5: Conclusion

Tocotrienols are naturally occurring bioactive compounds which possess anti-atherogenic and anti-cancer properties. In our pilot study with ESRD patients, TRF supplementation increased HDL, Apo-A1 and reduced CETP level, all which participate in latter steps of RCT. As a preliminary study of identifying potential benefits of TRF which will be given to ESRD patients in our multi-centered clinical intervention study, this study was designed to identify the anti-cancer properties of TRF and to explore the effects of TRF on cholesterol efflux, which is an early step of RCT. New TRF (TRF 2 and 3) significantly inhibited NSCLC cell growth indicating contents in TRF capsules are active. It further supported that new TRF have anti-cancer properties on NSCLC, whereas placebo did not. In addition, results suggest that TRF-2 acts as a potential PPAR-γ agonist, which may promote cholesterol efflux. Increased expression of PPAR-γ due to TRF-2 may cause no net uptake of cholesterol into macrophages through increasing CD36 gene and decreasing SRA1 genes. Further, TRF-2 increased the expression of SRB1 which may promote SRB1-mediated cholesterol efflux. Therefore, as a collective effect, TRF-2 may increase net cholesterol efflux. Taken together, data from this study suggest that TRF-2 which have a similar composition to TRF that will be given to ESRD patients, have potential anti-cancer effects and also may favor an increase in cholesterol efflux, reflecting its potential atheroprotective properties.

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## ABSTRACT

## EFFECTS OF PALM OIL TOCOTRIENOLS RICH FRACTION ON CHOLESTEROL EFFLUX

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Tocotrienols are naturally occurring bioactive compounds which possess beneficial effects on multiple chronic diseases including atherosclerosis and cancer. It is believed to exert atheroprotective effects via promoting reverse cholesterol transport, a process that removes excess cholesterol from the body. Cholesterol efflux is an early step in reverse cholesterol transport and impaired cholesterol efflux is known to trigger foam cell formation, a hallmark of atherosclerosis. An increase in cholesterol efflux and decrease in cholesterol influx in macrophages has been suggested to reduce atherosclerosis risk by reducing foam cell formation. In addition, tocotrienols have shown anti-cancer effects on Non-small cell lung cancer (NSCLC). Therefore, the objective of this study was to investigate time and dose dependent effects of Tocotrienols Rich Fraction (TRF) derived from Palm oil (74.68%

Tocotrienols and 23.5% Tocopherols) on PPAR-γ, a key modulator of cholesterol efflux, its downstream cholesterol influx (SRA1, CD 36) and genes related to efflux (LXR-α, ABCA1, ABCG1, SRB1) in J774A.1 macrophages. To confirm contents of TRF capsules are active, anti-cancer effects of TRF on NSCLC cell lines were used. Effects of three TRF batches (TRF 1, TRF 2 and TRF 3) on A549 cell proliferation were determined using MTS assay (0,40,80,120 µg/ml). Western-blot analysis was used to test the effects of TRF 1 on Notch-1 expression, a protein involved in cancer cell proliferation. All three different batches significantly reduced A549 cell growth, starting from 80 µg/ml and TRF -1 dose dependently decreased Notch-1 protein expression. Non-toxic concentrations range of TRF-2 for J774A.1 cells were determined using MTS assay and the effect of TRF-2 on PPAR-γ, influx and efflux genes were explored using real-time PCR. Based on MTS assay results, concentrations of 0, 8, 16 µg/ml of TRF-2 were selected for 24 and 48 hour treatments for J774A.1 cells. According to PCR data, treatment with 16 µg/ml TRF-2 for 48 hours showed the highest significant increase of PPAR-γ, SRB1, and CD 36 expression, and the highest significant decrease in SRA1 and ABCA1 expression compared to the control under tested conditions. At these conditions, both LXR- α and ABCG1 were not significantly changed. Taken together, our data suggests that TRF impacts both influx and efflux genes via upregulation of PPAR-γ, such that the net effect favors increase in cholesterol efflux.

## AUTOBIOGRAPHIC STATEMENT

Jayawardana Arachchige Erandi Jayamini Shanika Jayawardana (Jayamini Jayawardana) obtained her Bachelors of Science degree from University of Colombo, Colombo, Sri Lanka, majoring Biochemistry and Molecular Biology. In the same year, she started her graduate studies in the department of Nutrition and Food Science, Wayne State University, Detroit, USA and is expecting to complete her studies in August 2017.