ABSTRACT

The Effects of Fish Oil Supplementation on Inflammation Markers in Chronic Kidney Disease Patients

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The purpose of this study was to investigate the effects of the daily consumption of fish oil, containing 2.4g of n-3 fatty acids (1400 mg Eicosapentaenoic acid + 1000 mg Docosahexaenoic acid), on pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α for 8 weeks in CKD patients, stages 2-5. One prevalent characteristic of all stages of CKD is excessive production of these pro-inflammatory cytokines. Fish oil supplementation has been claimed to lower the levels of these pro-inflammatory cytokines, and as a result decrease the severity of inflammatory diseases. The benefits of fish oil supplementation for an extensive range of populations and a variety of health concerns are apparent, yet the anti-inflammatory benefits for stages 2-5 CKD patients are not as well documented. Consequently, continued studies in this area are clearly needed. Thirty-one individuals completed the current study, with 17 subjects in the fish oil group, while 14 subjects were included in the comparison group (safflower oil). Separate Repeated Measures ANOVAs were used to measure changes in the primary outcome variables using a 2 (fish oil or safflower oil) x 2 (time points) design. Significance level was set at $p \leq 0.05$. The

results of this study showed that fish oil supplementation does not decrease plasma pro-inflammatory markers TNF-α, IL-6, and IL-1 β in CKD patients, stages 2-5. The analysis of TNF-α levels revealed no significant difference across time ($p = 0.92$) or between groups ($p = 0.94$). However, there was a group by time interaction, ($p = 0.03$). The analysis between IL-6 levels and treatment resulted in no significant difference across time ($p = 0.30$), between groups ($p = 0.15$), nor was there a significant group by time interaction with the trends across time differing by group membership ($p = 0.82$). Finally, the analysis between treatment and IL-1β resulted in no significant difference in IL-1β levels across time ($p = 0.17$), between groups ($p = 0.26$) nor treatment-by-time interaction either ($p = 0.44$). The supplementation of fish oil was not found to decrease markers of inflammation, but did demonstrate that a short-term administration of fish oil is well-tolerated by CKD patients, stages 2-5. But, further investigation is essential to better define the long-term impact of fish oil supplementation in this high-risk population.

The Effects of Fish Oil Supplementation on Inflammation Markers in Chronic Kidney Disease Patients

by

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A Dissertation

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LIST OF ABBREVIATIONS

ACTH- Adrenocorticotrophic Hormone

AD- Alzheimer disease

AA- Arachidonic Acid

ALA- Alpha-Linolenic Acid

CKD- Chronic Kidney Disease

COX - Cyclooxygenase

CRF- Chronic renal failure

CRP- C- Reactive Protein

CVD- Cardiovascular Disease

DHA- Docosahexaenoic Acid

EBNL- Exercise and Biochemistry Nutrition Lab

EPA- Eicosapentaenoic Acid

ESRD- End Stage Renal Dialysis

GFR- Glomerular Filtratoin Rate

HD- Hemodialysis

hs-ELIZA - High-Sensitivity Enzyme-Linked Immunosorbent Assay

JNK- c-Jun N-terminal kinases

LA- Linoleic Acid

LDL- Low-Density Lipoprotein

MAPK- Mitogen-Activated Protein Kinase

PD- Peritoneal Dialysis

PUFA- Polyunsaturated Fatty Acid

ROS- Reactive Oxygen Species

TNF-α – Tumor Necrosis Factor Alpha

TNFR1- TNF receptor one

Il-6- Interleukin Six

IL-1β- Interleukin One Beta

N-3- Omega Three Fatty Acids

N-6- Omega Six Fatty Acids

NF-κβ - Nuclear Factor Kappa Beta

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CHAPTER ONE

Chronic diseases, such as Cardiovascular Disease (CVD), are the single greatest cause of global mortality (Cannon et al., 1991; Park, Park, & Yu, 2005; Petersen & Pedersen, 2005). Many chronic diseases are associated with low-grade systemic inflammation as evidenced by a two to threefold increase in pro-inflammatory cytokines during the disease process. Cytokines, such as interleukins and lymphokines, are regulatory proteins that are released by cells of the immune system and operate as intercellular mediators in the assembly of an immune response. Examples of pro-inflammatory cytokines include tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β). An increased release of pro-inflammatory cytokines, i.e., TNF-α, IL-6, or IL-1β, has detrimental effects, such as contributing to atherosclerosis. Further, adipose tissue-derived TNF-α is known to activate nuclear factor-κβ (NF-κβ), which organizes inflammatory changes within the vascular tissue and is involved in skeletal muscle proteolysis (Cannon et al., 1991; Park et al., 2005; Petersen & Pedersen, 2005).

Systemic inflammation largely contributes to many of the co-morbid conditions that Chronic Kidney Disease (CKD) patients experience, such as fever, reduced appetite, muscle proteolysis, acute phase protein synthesis, and CVD. CVD, such as atherosclerosis, is known to be the leading cause of morbidity and mortality in CKD patients (Himmelfarb et al., 2007; Moreira et al., 2007). The severity of CKD is categorized into five stages, with stage 1 being the mildest and having few symptoms, while stage 5 is represented by severe illness with poor life expectancy and often includes

dialysis. Stage 5 CKD is also called established chronic kidney disease and is synonymous with end-stage renal disease (ESRD), chronic renal disease, or chronic renal failure (Schocken et al., 2008).

One possible solution to the problem of systemic inflammation in CKD patients involves the use of dietary omega 3 (n-3) fatty acids. Various authors have revealed that dietary supplementation of n-3 fatty acids, found in fish oil, can lower systemic inflammation by decreasing inflammatory markers, such as TNF-α, IL-6 and IL-1β (Ferrucci et al., 2006; Rasic-Milutinovic et al., 2007; Simopoulos, 2006). In addition, *in vivo* studies have reported n-3 fatty acids to have antithrombotic and antiarrhythmic properties and improve insulin sensitivity (Ferrucci et al., 2006; Simopoulos, 2006). In contrast, an abundance of omega-6 (n-6) fatty acids, such as arachidonic acid (AA), leads to oxidation of low-density lipoprotein (LDL), platelet aggregation, and interferes with the inclusion of n-3 fatty acids in the cell membrane of phospholipids, thereby increasing inflammation, thrombosis, and insulin resistance (Ferrucci et al., 2006; Simopoulos, 2006). Research authors suggest that the benefits of n-3 fatty acids are achieving a balance with n-6 fatty acids. The n-6/n-3 ratio should be approximately 3-5/1 rather than the ratio of 20-16/1, which is currently the composition of the Western diet (Burns et al., 2007; Simopoulos, 2006). Three common forms of n-3 fatty acids include eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA). ALA is known as the parent n-3 fatty acid because it can be metabolically converted to EPA (20:5n–3) and DHA (22:6n–3). Yet, the production of DHA and EPA comes predominately from algae in the ecosystem; therefore, when fish consume algae they become rich in DHA and EPA (Arterburn, Hall, & Oken, 2006).

Fish oil is widely sold in gelatin capsules, which includes a combination of EPA and smaller quantities of DHA, due to the higher potency of DHA (Vedin et al., 2008). In addition, a continual supply of EPA and DHA is needed because of the limited storage space in adipose tissue (Gerster, 1998). DHA is the most abundant n–3 fatty acid in membranes and is found in all organs. Only a small amount of ALA and EPA are present in tissues, with DHA normally exceeding EPA 5- to 30-fold in a majority of the organs (Gerster, 1998). ALA supplementation does not result in a significant accumulation of long-chain n-3 fatty acids in plasma, but dietary supplementation of EPA and DHA can lower plasma AA concentrations. When EPA and DHA are added to the diet they can replace the n-6 fatty acids in the membranes of virtually all cells, such as endothelial cells, and present anti-inflammatory effects (Arterburn et al., 2006; Gerster, 1998; Simopoulos, 2006). This decrease in inflammation is associated with a decrease in both serum cytokine concentrations and in the assembly of pro-inflammatory cytokines (Ferrucci et al., 2006; Simopoulos, 2002b; G. Zhao et al., 2007).

Researchers have reported that a low level of n-3 fatty acids in the blood of CKD patients most likely contributes to their many inflammatory co-morbid outcomes (Madsen, Christensen, Blom, & Schmidt, 2003; Rasic-Milutinovic et al., 2007; Saifullah et al., 2007). Furthermore, the American Heart Association recommends that patients with high risk for cardiovascular morbidity and mortality, such as CKD patients, consume at least 1g of fish oil daily, as well as maintain a dietary ratio of 4:1 for n-6 to n-3 fatty acids (Park et al., 2005; Petersen & Pedersen, 2005). Many studies, have been conducted in CKD 5 or ESRD patients with n-3 fatty acids, but not many have looked at the pro-inflammatory cytokines or less severe stages of CKD (Beavers, Beavers,

Bowden, Wilson, & Gentile, 2008; Donadio, Bergstralh, Bibus, & Grande, 2006; Lancaster, 2004; Svensson, Christensen, Solling, & Schmidt, 2004; Svensson, Schmidt, Jorgensen, & Christensen, 2007; Taziki, Lessan-Pezeshki, Akha, & Vasheghani, 2007). In the few studies that have been conducted in CKD patients, researchers have found positive results regarding a decrease in inflammatory markers. For example, Perunicic-Pekovic et al. (2007) reported a significant decrease in IL-6 and TNF-α levels after eight weeks of supplementing 2.4 g a day of n-3 fatty acids (EPA + DHA). Similarly, Cappelli Di Liberato, Stuard, Ballone, and Albertazzi (1997) found a decrease in IL-1β, IL-6, and TNF-α after 12 months in ESRD patients ingesting 3.4 g of n-3 fatty acids (EPA + DHA). Further, ESRD patients that supplemented fish oil supplements for eight weeks found a significant decrease in IL-6 (Himmelfarb et al., 2007). In a variety of other populations, study authors have found mixed results in supplementing patient's diets with n-3 fatty acids. For example, Alzheimer disease (AD) patients and healthy populations have reported decreases in these inflammatory markers (Endres et al., 1989; Espersen et al., 1992; Meydani et al., 1991a; Sundrarjun et al., 2004; Tsitouras, Gucciardo, Salbe, Heward, & Harman, 2008) while other populations, such as rheumatoid arthritis patients and type 2 diabetics did not find a decrease in either TNF-α or IL-6 (Espersen et al., 1992; Mori et al., 2003; Vedin et al., 2008).

With limited studies examining the effects of fish oil supplementation on inflammatory markers in CKD patients, it is clear that further research is needed to elucidate their purported benefits. Further, how n-3 fatty acids effect pro-inflammatory cytokines within the systemic circulation is also unclear.

Purpose of the Study

The purpose of the current study was to investigate the effects that the daily consumption of 2.4 g of n-3 fatty acids (1400 mg EPA + 1000 mg DHA) has on pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α for eight weeks in CKD patients, stages 2-5.

Hypotheses

H_1

There will be a decrease in plasma levels of TNF-α following eight weeks of fish oil supplementation in stages 2-5 of CKD patients.

H_2

There will be a decrease in plasma levels of IL-6, following eight weeks of fish oil supplementation in stages 2-5 of CKD patients.

H_3

There will be a decrease in plasma levels of IL-1β following eight weeks of fish oil supplementation in stages 2-5 of CKD patients.

Delimitations

This study was completed using the following guidelines:

1. Fifty CKD Patients, stages 3-5, and ≥ 18 years participated in this study. Power calculations showed that 20 participants per group were necessary to detect a significant difference between groups in inflammatory markers given a type I error rate of 0.05 and a power of 0.80. Therefore, 10 patients were added in case of non-compliance.

2. Participants were recruited from the Central Texas Nephrology Associates in conjunction with Dr. Ronald L. Wilson and Family Practice Clinic in conjunction with Dr. Griggs, both in Waco, Texas. Dr. Wilson and Dr. Griggs provided recruitment flyers to their patients.
3. Participants were randomly assigned to one of two supplementation groups: "Super-Omega- 3" or the comparison (safflower oil) group.
4. Fasting venous blood was collected at the start of the study, four weeks after initiation, and then eight weeks after initiating the study.
5. All participants in the study took four capsules everyday for eight weeks and did not modify their nutritional intake in any manner.
6. All blood samples were analyzed in the Exercise and Biochemical Nutrition Lab at Baylor University in the Marrs-McLean Gym according to all policies and procedures within the laboratory.

Limitations

1. The sample size was limited to those who came forward to participate in the study, which limited the scope of conclusions that can be inferred to a larger population.
2. The motivation and willingness of each participant to be compliant with supplementation.
3. The sensitivity of the technologies and protocols utilized to identify quantifiable changes in the criterion variables.

4. The daily schedules of each participant and the inherent circadian rhythms that exist for all humans as a result of slightly different testing times, stresses, dehydration, etc.

Assumptions

1. All participants followed all guidelines and consumed the prescribed dose of fish oil or placebo for the duration of the study.
2. Participants fasted at least eight hours prior to reporting for blood sampling.
3. Participants maintained close contact with primary investigators and other research assistants during the study.
4. All participants maintained their regular dietary habits throughout the study and arrived at every scheduled session.
5. All assay reagents and equipment used in the sample analysis were accurate and reliable in quantification of the criterion variables.
6. All methods previously established are accurate and reliable methods for determination of the criterion variables.

CHAPTER TWO

Review of Literature

Chronic Kidney Disease Patients

Evidence suggests that over 19 million Americans have CKD, and millions of others are at increased risk (Ramos, Shintani, Ikizler, & Himmelfarb, 2008). Patients at all stages of CKD (1-5) experience elevated levels of inflammation and have a high morbidity and mortality rate, largely as a result of Cardiovascular Disease (CVD). Patients with earlier stages (stage 1-2) of CKD are twice as likely to have CVD and it advances at twice the rate when compared to the general population (Kovesdy & Kalantar-Zadeh, 2008; Yao, Axelsson, Stenvinkel, & Lindholm, 2004). Increased inflammation in CKD is highly linked to both all-cause and cardiovascular mortality. Therefore, having a successful treatment for chronic inflammation may improve long-term survival in these patients (Zimmermann, Herrlinger, Pruy, Metzger, & Wanner, 1999).

CKD results in a progressive loss of kidney function over a period of months or years. The functions of the kidneys include regulating total body water, sodium, potassium, phosphorus, and calcium. Kidneys are also used to remove drugs and toxins that could cause damage to tissues. In addition, the kidneys help to release hormones, rennin, erythropoietin, and calcitriol, which regulate blood pressure, produce red blood cells, and promote strong bones, respectively (Bailie, Uhlig, & Levey, 2005). Therefore, when kidney function decreases, blood pressure increases due to an overload of fluid in the blood, increasing the risk of developing hypertension, congestive heart failure, or life

threatening pulmonary edema. Uremia and hyperkalemia will also occur, which is an accumulation of urea or potassium in the blood, respectively. These increases come with symptoms ranging from lethargy to pericarditis or potentially fatal cardiac arrhythmias. Erythropoietin synthesis is also decreased, and therefore anemia develops, leading to fatigue. Further, metabolic acidosis often occurs, due to accumulation of sulfates, phosphates, and uric acid, causing a problematic shift in enzyme activity (Bailie et al., 2005; Levey et al., 2003). The most common risk factors for CKD include high blood pressure, diabetes or having a blood relative with kidney disease. Symptoms that indicate kidney dysfunction are vague, but most often include malaise and a decreased appetite (Bailie et al., 2005; Levey et al., 2003).

CKD is identified by either a blood or urine test demonstrating high levels of creatinine or urea. Creatinine is a product of normal muscle breakdown and urea is the waste product from the breakdown of protein. The amount of creatinine and urea excreted in the urine or found in the blood can be used to calculate the level of kidney function and the glomerular filtration rate (GFR- rate at which the kidneys filter blood). Therefore, when the levels of these substances rise, this indicates that the GFR has decreased. This decreased rate inhibits the kidney's ability to eliminate waste products, causing the symptoms to occur listed above and is classified by stages 1-5. The classification system uses the progressive decrease in GFR. Therefore, a higher stage of CKD indicates a decrease in the GFR. The normal GFR is about 100-140 mL/min in men and 85-115 mL/min in women (Delanghe et al., 1989). Patients with stage 1 CKD have normal GFR, but have abnormalities such as proteinuria, which is an excess of serum proteins in the urine. More advanced losses of GFR are classified as stage 2

(estimated GFR 60-90 cc/min), stage 3 (estimated GFR 30-60 cc/min), stage 4 (estimated GFR 15-30 cc/min), to stage 5 with less than 15 cc/min with or without dialysis therapy. Patients that are considered stage 5 are known as having End Stage Renal Disease (ESRD). ESRD patients may require renal replacement therapy; such as dialysis or ideally a kidney transplant (Bailie et al., 2005). Dialysis treatments replace some of the functions of the kidneys through waste and fluid removal, but it does not correct the endocrine functions of the kidney. There are two primary types of dialysis, hemodialysis (HD) and peritoneal dialysis (PD). In HD, the patient's blood is pumped through the blood compartment of a dialyzer, exposing it to a semi-permeable membrane. The cleansed blood is then returned by a circuit back to the blood flow, usually through the forearm. In PD, a sterile solution containing minerals and glucose is run through a tube into the peritoneal cavity, the abdominal body cavity around the intestine, where the peritoneal membrane facilitates as a semi-permeable membrane (Bailie et al., 2005; Levey et al., 2003; Sarnak et al., 2003). Both are imperfect solutions and unfortunately there is no specific treatment explicitly shown to slow the progression of CKD. But if inflammation is treated, which is a known cause of CKD, then it is possible to slow down the damage and delay further complications (Levey et al., 2003).

Inflammation

Inflammation is a typical endogenous response to harmful stimuli, such as pathogens, irritants, or cells that have been damaged. This defense system is used to eliminate stimuli that will cause further destruction to tissues, such as the endothelium, and then helps in the healing process. Common characteristics of inflammation include redness, swelling, heat, and pain. These occur as a result of elevated body flow and an

increased number of leukocytes moving from the bloodstream to the adjacent tissue. Permeability is also increased across blood capillaries, allowing cytokines to leave the bloodstream and cross the endothelial wall (Calder, 2007).

Acute inflammation is the first response to destructive agents, such as pathogens or irritants, and occurs with an increase in plasma and leukocytes moving from the blood to the injured site. Conversely, chronic inflammation is prolonged inflammation that leads to a progressive change in the type of cells that are found at the site of inflammation (Calder, 2007). The main reason for the switch from acute to chronic inflammation is the recruitment of monocytes to the area of inflammation with the help of IL-6, a well known cytokine (Kaplanski, Marin, Montero-Julian, Mantovani, & Farnarier, 2003). Inflammation is necessary for the healing of wounds and infections, however chronic inflammation is very damaging and is chronically elevated in numerous acute and chronic human diseases (Wanner, Zimmermann, Schwedler, & Metzger, 2002). For example, CVD is linked to low-grade systemic and chronic inflammation, which is not confined to a particular tissue but involves the endothelium and other organ systems. Systemic inflammation is recognized by an elevation in the amount of pro-inflammatory cytokines, arachidonic acid (AA), adhesion molecules, and additional inflammatory agents, such as reactive oxygen species (ROS) (Calder, 2006).

The initial cells to arrive at chronic inflammation sites include granulocytes, monocytes, macrophages, and lymphocytes. These initial cells are used to destroy pathogens, remove cellular and tissue debris, and promote tissue repair. One significant exogenous trigger is bacterial endotoxin, which can directly stimulate monocytes and macrophages, inducing them to form pro-inflammatory cytokines, such as TNF-α, IL-6,

and IL-1ß. Pro-inflammatory cytokines initiate an increase in inflammatory mediators, such as resolvin, and amplify the first inflammatory signal (Kofler, Nickel, & Weis, 2005; Simopoulos, 2002b). However, in CKD patients, sustained elevations of pro-inflammatory cytokines play a key role in the pathogenesis of kidney disease and its progression to ESRD, with the amount of pro-inflammatory cytokines correlating with disease severity (Guebre-Egziabher & Fouque, 2003). High concentrations of TNF-α, IL-6, and IL-1ß are particularly damaging because they increase oxidative stress and induce endothelial cell apoptosis, causing vascular disease (Kofler et al., 2005; Simopoulos, 2002b). Pro-inflammatory cytokines increase when renal function begins to decline, suggesting that CKD is a low-grade systemic inflammatory process that enhances renal function deterioration (Cachofeiro et al., 2008). Therefore, systemic inflammation is a common feature of CKD, even before the start of renal replacement therapy. HD patients and CKD patients, not yet on dialysis, have been found to have significantly higher levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1ß, when compared to an apparently healthy population. For example, a study conducted by de Vinuesa, compared 52 CKD patients, stages 3 and 4 CKD and no diabetes, with healthy control subjects (de Vinuesa et al., 2006). Patients with CKD presented increased levels of TNF- α and IL-6, demonstrating that high amounts of inflammation are found even before dialysis.

In CKD patients, there are multiple causes for increasing plasma levels of pro-inflammatory cytokines, including non-dialysis as well as dialysis-related factors. The kidney is the main site for the removal of many cytokines, yet, pro-inflammatory cytokines and their inhibitors are markedly distorted in CKD patients. This results in a

state of chronic inflammation, which is common in all stages of CKD (Carrero, Yilmaz, Lindholm, & Stenvinkel, 2008; Qureshi et al., 1998; Yeun & Kaysen, 2000). Other causes of inflammation include fluid overload, oxidative stress, increased susceptibility to infection, and the presence of co-morbid conditions, such as diabetes or hypertension. Specifically in ESRD patients, reasons for inflammation include the exposure to dialysis tubing and dialysis membranes; poor quality of dialysis water, back-filtration, or back-diffusion of contaminants; and foreign bodies in dialysis access (Kalantar-Zadeh, Stenvinkel, Pillon, & Kopple, 2003; Stenvinkel et al., 2005; Stenvinkel, 2006; Wang, 2005).

The cytokine cascade can itself account for fever, reduced appetite, muscle proteolysis, lipolysis, and acute phase protein synthesis, which are major predictors of poor clinical outcome in kidney failure (Kalantar-Zadeh et al., 2003; Pertosa, Grandaliano, Gesualdo, & Schena, 2000; Stenvinkel, 2006; Wang, 2005). It has been indicated that the relationship between inflammation and nutrition is unquestionably bidirectional with inflammation disturbing nutritional status and dietary factors controlling the state of inflammation. TNF- α, along with IL-6 and IL-1β, will interfere with the satiety center causing a loss of appetite and delayed gastric emptying, which results in malnutrition (Kuhlmann & Levin, 2008). Data suggests that higher inflammatory markers in malnourished CKD patients will classify them at high risk of co-morbidity and mortality (Kaysen, 2004; Stenvinkel & Alvestrand, 2002). Further, the pro-inflammatory cytokines can cause muscle weakness by at least two mechanisms, increased protein loss and contractile dysfunction. This loss of protein is a chronic response that can occur over days to weeks (Reid & Li, 2001).

Notwithstanding, the cardiovascular system is one of the main targets of inflammation. There is a strong association between systemic inflammation and coronary artery disease. This association is thought to be causal, such that inflammation increases the risk of the disease, rather than simply marking the presence of atherosclerosis, which is an inflammatory process (Ross, 1999). The initial stage of atherosclerosis in CKD patients begins with an insult to the vascular endothelium. Monocytes and macrophages are attracted to the altered endothelium and therefore infiltrate the vessel wall to initiate the inflammatory process. Thus, cytokines are commonly higher in people with CKD, chronic coronary artery disease and peripheral vascular disease, when compared with apparently healthy populations (Browning et al., 2007; Calder, 2007; Maruyama, Stenvinkel, & Lindholm, 2005; Petersen & Pedersen, 2005). Patients with CKD are at high risk from potentially devastating CVD, such as atherosclerosis, due to the unique clustering of risk factors in these patients. Renal failure and CVD have actually been noted as expressions of the same disease process. For example, many of the risk factors, such as diabetes, dyslipidemia, or hypertension, are risk factors for CKD, ESRD, and CVD (Kaysen, 2004). These risk factors can cause endothelial dysfunction characterized by off-balanced vasodilatation and vasoconstriction, increased oxidative stress and inflammation, deregulation of thrombosis and fibrinolysis, abnormal smooth muscle cell proliferation, and a deficient repair mechanism. But, these risk factors cannot fully explain the reason for extremely high cardiovascular mortality in CKD. However, it has recently been discovered that it is chronic inflammation that contributes to the increased mortality rate seen in patients with CKD. Therefore, it has been said that high levels of TNF- α and IL-6 powerfully predict death from CVD (Wu-Wong, 2008). Wanner et al.

(2002) conducted a study with HD patients that had high levels of inflammation, determined by C- reactive protein (CRP), an acute-phase protein. They found that after four years, approximately 44% had died, with the death rate curve found to be linear at 11% per year, 58% of the deaths occurring from cardiovascular events (Wanner et al., 2002). This study confirms that inflammation is associated with an increased overall and cardiovascular mortality in HD patients and adds data that prediction is even maintained after prolonged periods of observation.

Tumor Necrosis Factor Alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine involved in systemic inflammation and is mainly produced by macrophages along with many other cells, such as skeletal muscle cells and adipocytes (Hotamisligil, Shargill, & Spiegelman, 1993). TNF-α, along with IL-1β, are the most studied cytokines and are first in the cytokine cascade. The primary roles of TNF-α include regulation of immune cells and stimulating apoptotic cell death to generate inflammation and prevent tumorigenesis and viral replication (Petersen & Pedersen, 2005). However, an overproduction of TNF-α has been linked to many chronic diseases (Calder, 2007). For example, TNF-α is the first cytokine recognized to have an indirect role in promoting insulin resistance by increasing the release of free fatty acids from adipose tissue (Calder, 2007; Park et al., 2005). Perunicic-Pekovic et al. (2008) found that subjects with risk genotypes for both TNF- α and IL-6 have the highest incidence of diabetes, which is a risk factor for CKD. Given that TNF- α mainly works locally, TNF-α transcription may not always be reflected in enhanced systemic levels of TNF- α. Instead, TNF- α will stimulate IL-6 production and consequently, CRP. Therefore, chronically elevated levels of IL-6 and CRP are likely to reflect local ongoing

TNF- α production (Petersen & Pedersen, 2005). In a study conducted by Espinoza et al. (1999), TNF-α plasma levels were evaluated in 49 stable PD patients with the average plasma level of TNF-α was 67 +/- 32 pg/mL (range: 18.1-156.3 pg/mL; normal value 3-20 pg/mL). TNF- α was found higher in patients with altered creatinine levels as compared to patients with normal creatinine levels. In addition, patients with hypertriglyceridemia or taking lipid-lowering agents showed a positive linear correlation between TNF-α and triglycerides (Espinoza et al., 1999). This data implied that an increase in TNF-α may lead to the formation or maintenance of some neurologic, hematologic, and cardiac complications in PD patients. Loss of residual renal function, which is linked to altered creatinine levels, causes an increase in TNF-α levels. Further, this supports the idea that TNF-α may be considered a uremic toxin.

TNF-α applies its cellular effects by binding to specific receptors, namely TNF receptor-1 (TNFR1) and TNFR2. Then, TNF-α promotes an assortment of post-receptor signaling events, predominantly through three major pathways: a) an apoptotic signaling pathway, b) activation of JNK and MAPK pathway, and c) activating transcription factor nuclear factor-κB (NF-κB) pathway (Uysal, Wiesbrock, Marino, & Hotamisligil, 1997). Alterations in gene expression necessary for TNF-α to induce catabolism is regulated by NF-κB, which contributes to the net loss of muscle protein caused by chronic TNF-α exposure. Conversely, contractile dysfunction is an acute response to TNF- α stimulation, causing decreased force production. Both actions of TNF-α entail a quick rise in endogenous oxidants, such as reactive oxygen species (ROS), which is an essential step in post-receptor signal transduction (Reid & Li, 2001). This provides information into the cellular and molecular mechanisms of TNF- α action in skeletal muscle.

Interleukin Six (IL-6)

Interleukin Six (IL-6) is produced predominantly by macrophages as well as adipocytes in various tissues and is a plieotrophic cytokine, that is, it has both an anti- and pro-inflammatory effect. Therefore, production in the right amounts can be useful in responding to infection, but in inappropriate amounts can be damaging, producing pro-inflammatory conditions. When IL-6 acts as an anti-inflammatory cytokine, it can prevent TNF-induced insulin resistance. This most likely occurs due; in part, to the release of soluble TNF receptors (Tilg, Trehu, Atkins, Dinarello, & Mier, 1994). Yet, when IL-6 exerts pro-inflammatory effects, it can cause stimulation of the liver to produce positive acute-phase proteins during tissue injury or infection. It can also induce fever; activate T and B lymphocytes, and endothelial cells. At the beginning of acute inflammation, IL-6 mediates the acute phase responses. IL-6 is the only cytokine that can stimulate the synthesis of all the acute phase proteins involved in the inflammatory response, such as CRP. When its activity as a pro-inflammatory cytokine persists, acute inflammation turns into chronic inflammation that includes immune responses. In chronic inflammation, IL-6 has a damaging role that favors mononuclear cell accumulation at the site of injury, which magnifies chronic inflammatory proliferation (Simopoulos, 2002b). As a result, IL-6 is reported to play a key role in the pathophysiology of the destructive effects of inflammation in CKD patients (Simopoulos, 2002b). Further, it is has been found that pro-inflammatory cytokines, TNF-α and IL-1β, stimulate endothelial cells to produce IL-6 (De Caterina, Cybulsky, Clinton, Gimbrone, & Libby, 1995; Pepys & Hirschfield, 2003; Simopoulos, 2002b). Therefore, researchers suggests that a combined elevation of IL-1β, TNF-α, and IL-6, rather than the IL-6 alone,

increases the risk factors for CKD, such as type 2 diabetes (Reiner, Tedeschi-Reiner, & Stajminger, 2007).

A decline in renal function, as related to an increase in cytokines, such as IL-6, may be somewhat to blame for the rise in CRP, a common marker of inflammation used in CKD patients. Therefore, IL-6 is most likely a better indicator of mortality and inflammation than CRP (Panichi et al., 2004; Reiner et al., 2007). To confirm the accuracy of using IL-6 as an evaluation marker, several studies have been conducted. For example, Panichi et al. (2004) realized that even though there is a well known association between IL-6 and cardiovascular mortality, no study had confirmed whether IL-6 adds prognostic information to that provided by CRP. Plasma IL-6 and CRP concentrations were assessed in a cohort consisting of 218 HD patients from four different dialysis centers. Full information on co-morbidities was available in 162 patients. After full evaluation, it was concluded that IL-6 is a stronger predictive value than CRP for cardiovascular mortality and provides independent prognostic information, while conveying most of that provided by CRP (Panichi et al., 2004). Another study had 176 patients with ESRD (54 +/- 12 years) undergo measurements of serum albumin (S-Alb), CRP, and plasma IL-6 close to the start of dialysis therapy and were followed up in a range of 1 to 66 months. Nutritional status was evaluated by means of subjective global assessment and CVD was defined based on medical history. All biomarkers predicted malnutrition, CVD, and mortality. Analyses showed that in patients with ESRD, malnutrition is predicted best by CRP and IL-6 levels; CVD, by IL-6 levels; and mortality, by S-Alb and IL-6, but not by CRP levels. This comparative analysis indicated

that of these biomarkers, IL-6 level may be the most reliable predictor of CVD and mortality in patients with ESRD (Honda et al., 2006).

Further, Herbelin, Urena, Nguyen, Zingraff, & Descamps-Latscha (1991) attempted to determine the respective influence of HD and uremia on the plasma level IL-6, which shares several biological properties with IL-1β and TNF-α. Forty-eight patients with ESRD, including 32 long-term HD patients and 16 chronic uremic patients undergoing their first dialysis session, were tested for plasma IL-6 using both biological and immunoreactive assays. Plasma IL-6 activity was significantly higher in patients with chronic CKD when compared to apparently healthy individuals. No difference was observed, however, between long-term and not yet dialyzed patients. In the patients with the most pronounced IL-6 activity, immunoreactive IL-6 levels were between 60 and 150 pg/ml (Herbelin et al., 1991), while normal values found less than 10 pg/mL (Mori et al., 2003). No change in plasma IL-6 was detected during the course of the first dialysis session, as well as subsequent sessions (Herbelin et al., 1991). Secondly, circulating plasma IL-6 was evaluated as a predictor of all-cause and cardiovascular mortality and its relationship to prevalent comorbidity and hypoalbuminemia, in a cohort of stable HD patients. Clinical data included demographic, medical, and routine laboratory parameters. Blood samples were taken at enrollment and annually, and plasma IL-6 levels measured with high-sensitivity enzyme-linked immunosorbent assay (hs-ELIZA). Median plasma IL-6 level in 206 patients was 7.9 pg/mL (range - 0.1 to 90.3 pg/mL) and was found higher in patients with vascular disease. Unadjusted median survival time was 1,209 days in the lowest quartile of plasma IL-6 and 806 days in the highest (Rao et al., 2005). Taken together, these results demonstrate that plasma IL-6 levels are strongly

associated with co-morbidity in HD patients and are a powerful predictor of all-cause and cardiovascular mortality (Rao et al., 2005). A similar study was conducted to determine whether or not plasma IL-6 can predict patient survival. One hundred and seventy-three ESRD patients were studied near the initiation of dialysis treatment (99 PD, 74 HD patients). The patients were followed for a mean period of three years and were placed into groups at the start of dialysis treatment according to age, gender, presence of CVD, malnutrition, diabetes mellitus, and IL-6 plasma levels. When patients were stratified according to IL-6 quartiles and analyzed separately according to the different initial treatment groups, a similar profile of survival was observed for PD and HD patients (Pecoits-Filho, Barany, Lindholm, Heimburger, & Stenvinkel, 2002). This study confirmed that the strong predictive value of elevated IL-6 levels for poor outcome in ESRD patients is similar in both HD and PD patients starting treatment.

The cross-sectional association of renal insufficiency with inflammatory markers was evaluated using baseline data from the Cardiovascular Health Study, a population-based cohort study of approximately 6000 subjects aged $\geq$ 65 years. IL-6 was among other inflammatory markers that were measured in roughly half of the participants. Renal insufficiency was defined as a serum creatinine level $\geq$ 1.3 mg/dL in women and $\geq$ 1.5 mg/dL in men and was found present in 11% of the participants. After adjustment for baseline differences, IL-6 levels were significantly greater among those with renal insufficiency. In participants with clinical, subclinical, and no CVD at baseline, the positive associations of renal insufficiency with this inflammatory marker was similar at baseline (Shlipak et al., 2003). Therefore, this marker may be an important mediator leading to the increased cardiovascular risk of persons with kidney disease.

Interleukin One Beta (IL-1β)

IL-1β is one of the most important soluble mediators of inflammation and is one of the molecular forms of IL-1, a potent pro-inflammatory cytokine. IL-1β is produced by macrophages, monocytes and dendritic cells, and mediates a wide range of reactions involved in the acute phase response (Calder, 2006; Maruyama et al., 2005). IL-1β increases the expression of adhesion factors on endothelial cells to enable movement of leukocytes, the cells that fight pathogens, to sites of infection. It also resets the hypothalamus thermoregulatory center, leading to an increased body temperature which expresses itself as fever. Minimal levels of IL-1β can induce fever, hypotension, and the release of adrenocorticotrophic hormone (ACTH) and the formation of cytokines, such as IL-6. IL-6 then induces the synthesis of CRP and stimulates the synthesis of adhesion molecules in endothelial cells and leukocytes. IL-1β also alters the normal blood compatible surface of the endothelium, causing coagulation and thrombosis while impeding fibrinolysis (Cappelli et al., 1997). In a study conducted by Pereira et al. (1994), plasma levels of IL-1β and TNF-α were measured in 29 undialyzed patients with CKD, 13 patients on PD, 42 patients on HD, and 15 apparently healthy controls. Of the 29 patients with CKD, 13 had ESRD. In the healthy controls, plasma levels of IL-1β and TNF-α were at or below the limit of detection of the assay. In undialyzed patients with ESRD, and in patients on PD and HD, plasma levels of IL-1 β were found at much higher than healthy controls (428 +/- 134 pg/ml, 378 +/- 83 and 352 +/- 43 pg/ml, respectively) (Pereira et al., 1994). The normal range when testing plasma samples in healthy donors is < 14 pg/ml (Espersen et al., 1992). Therefore, this gives evidence to the theory that IL-1β is usually found at higher elevations in CKD patients

Omega-3 (n-3) Fatty Acids

The increased rate of inflammatory diseases in Western countries is possibly due to a high consumption of saturated fatty acids and n-6 fatty acids and an insufficient intake of n-3 fatty acids in the diet. Epidemiological studies propose an inverse relationship between the intake of n-3 fatty acids and the incidence of CVD, at least in part due to reduced atherosclerosis, which is an inflammatory mediated process (De Caterina et al., 1995). Therefore, a suggested treatment for inflammation is the dietary supplementation of n-3 fatty acids. N-3 fatty acids have been a key element in the human diet since the beginning of time, yet for the past 150 years it has been shown that n-3 fatty acids have decreased in the Western diet, due to food processing and decreased fish intake (Simopoulos, 2002b). Since this need has been revealed, industries have been producing n-3 enriched products. N-3 fatty acids are an example of Polyunsaturated Fatty Acids (PUFAs), which are long chains of carbons, with two or more double bonds, and are the essential components of cell membranes, as shown in Figure 1.

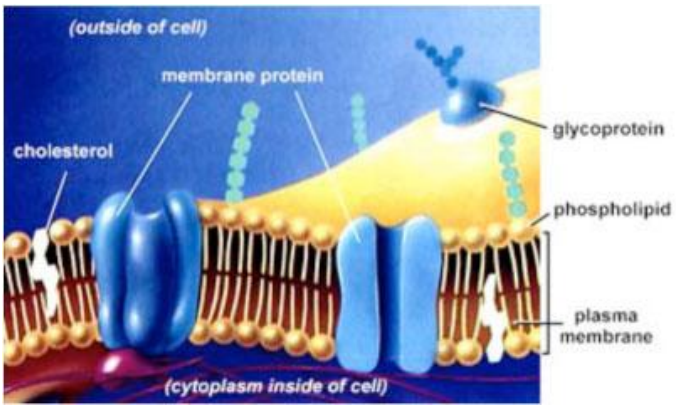

Figure 1. PUFAs (phospholipids) in the cell membrane (Friedman & Moe, 2006).

They determine cellular membrane fluidity and control enzyme activities for carriers and membrane receptors. These fatty acids are recognized as essential because they cannot be produced endogenously and can only be obtained from the diet (Simopoulos, 2002b).

Two well-known PUFAs are linoleic acid (LA), from the n-6 family and ALA of the n-3 family. In n-3 fatty acids, the position of the first double bond is located 3 carbons from the methyl end and n-6 is 6 carbons away, as shown in Figure 2.

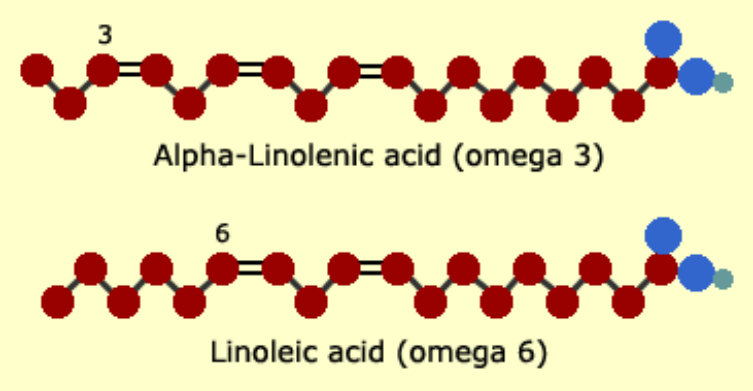

Figure 2. Difference in structure between n-3 and n-6 fatty acids (Gerster, 1998)

Through enzymatic steps, there is carbon chain elongation and desaturation of these acids, forming longer chain metabolites. For example, LA is converted to AA, and ALA is converted to EPA and DHA, which all can also be found in the diet. The conversion of ALA to EPA and DHA takes place predominantly in the endoplasmic reticulum of the liver and entails a series of elongation enzymes that sequentially add 2-carbon units to the fatty acid backbone and desaturation enzymes that insert double bonds into the molecules (Gerster, 1998). AA is found in red meat, which is a large proportion of the Western diet. DHA and EPA originate in cold-water fish, which is more often found in a Mediterranean diet (Friedman & Moe, 2006; Simopoulos, 2002b). EPA and DHA are shown to suppress

the capacity of monocytes to synthesize IL-1β mRNA, IL-1β, and TNF-α and the production of IL-6 by venous endothelial cells. These results may suggest a possible therapeutic role from supplementation of n-3 fatty acids in chronic inflammatory diseases (Calder, 2007; James, Gibson, & Cleland, 2000).

PUFAs have many important roles; such as generating eicosainoids. Eicosanoids are signaling molecules that control a range of bodily functions, predominantly in inflammation or immunity, and serve as messengers in the central nervous system. The four families of eicosainoids include prostagalndins, thromboxanes, leukotreines, and prostacyclins. It has been established that eicosanoids from n-6 and n-3 fatty acids have opposing properties (Calder, 2006). Eicosanoids produced from AA (n-6) have roles in inflammation and regulate T and B lymphocyte functions. Conversely, the n-3 family has anti-inflammatory roles, with EPA giving rise to eicosanoids and DHA to docosanoids. EPA and DHA-derived compounds function to dilate blood vessels; prevent platelet aggregation; lower arterial pressure; and inhibit thrombosis, cholesterol synthesis, and inflammation, as shown in Figure 3. Dietary intake of fatty acids is the main determinant of eicosanoid metabolism and the composition of fatty acids available in the anti-inflammatory structure of the cell membrane establishes which class of eicosanoid by-products will predominate (Calder, 2006; Fiedler, Mall, Wand, & Osten, 2005). A reduction in the generation of AA–derived mediators that accompanies fish oil consumption has led to the thought that fish oil, an n-3 fatty acid, is anti-inflammatory (Nader et al., 2006). Therefore, it is important to note the balance of specific fats in the diet and inflammatory responses (Calder, 2006; Fiedler et al., 2005). EPA and DHA also give rise to newly discovered resolvins, which are compounds produced by the

cyclooxygenase (COX-2) pathway that help to decrease cellular inflammation. In particular, resolvin E1 has been found to curb the activation of NF-κB, which affects inflammatory conditions in vascular tissue and is related to proteolysis in skeletal muscle. This resolvin also has influence on decreasing the production of pro-inflammatory cytokines, such as IL-1β and TNF-α (Friedman & Moe, 2006). Human immune cells are typically plentiful in AA; as a result it is imperative to increase EPA and DHA in the diet in order to change the pattern in the production of eicosanoids, docosanoids, and resolvins (Calder, 2007).

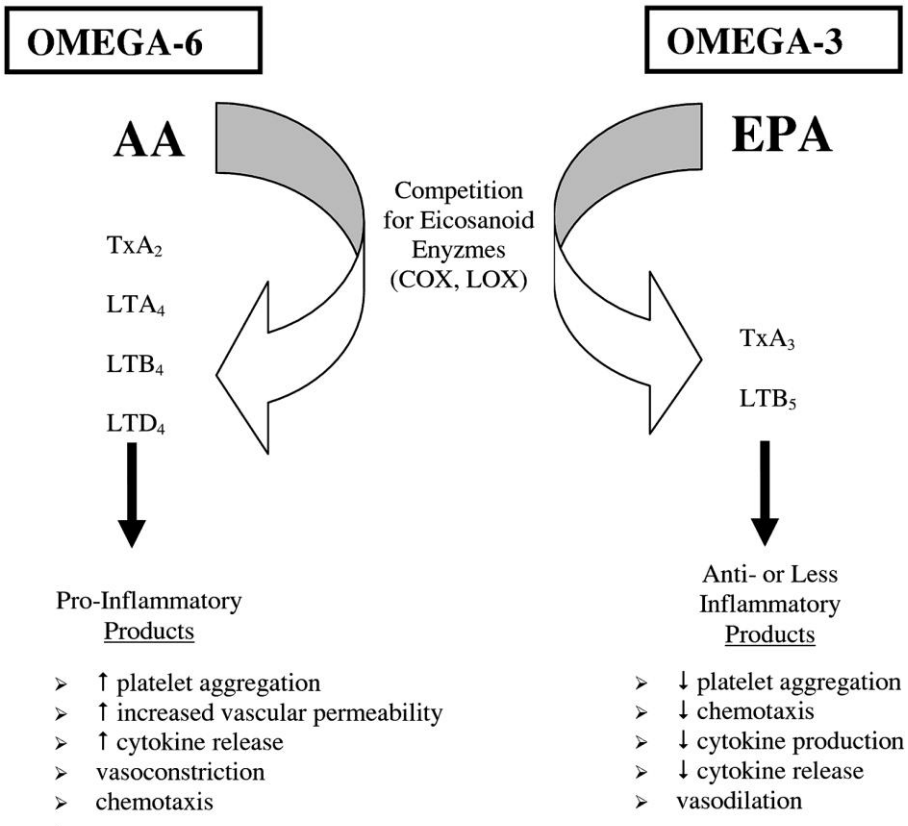

Figure 3. The difference between n-6 and n-3 in inflammation (Friedman & Moe, 2006). DHA: Docosahexanoic Acid, LOX: Lipoxygenase, EPA: Eicosapentanoic Acid, TxA: Thromboxane, AA: Arachidonic Acid, LT: Leukotriene, COX: Cyclooxygenase

In regards to CKD patients, it is common to see low levels of n-3 fatty acids in their blood, which can lead to a multitude of problems. Examples include dialysis access thrombosis, atherosclerosis, insulin resistance, and dyslipidemia. Reasons for low levels of n-3 in CKD patients may be because they find the major food sources of EPA and DHA to be less palatable, possibly caused by an uremia-associated alteration in taste. Also they might be limited financially, by social dietary habits or they follow the typical Western diet, which includes higher amounts of saturated fat and n-6 fatty acids (Calder, 2006; Engler & Engler, 2006). In the few studies that have evaluated the pro-inflammatory cytokines in CKD patients taking fish oil, they have been focused on ESRD patients and therefore studies in patients with CKD stages 3 and 4 are lacking. But in ESRD patients, positive changes in inflammation markers have been observed. Rasic-Milutinovic et al. (2007) explored the effects of 2.4g n-3 fatty acid supplementation (180 mg of EPA and 210 mg of DHA) on the inflammatory markers TNF-α and IL-6 for eight weeks. At the end of the study, it was concluded that a moderate dose of EPA and DHA in CKD patients on HD will significantly decrease IL-6 and TNF-α after eight weeks. Further, it was found to decrease insulin resistance. Another study found a correlation between markers of inflammation and parameters of malnutrition in HD patients. Forty-two HD patients were evaluated, at the mean age of 55 years. Baseline values in the tested group confirmed the presence of essential fatty acids deficiency. They were compared to a control group that consisted of 16 apparently healthy subjects of similar age and sex. At the start of the study, the HD patients with severe malnutrition had higher levels of IL-6 and TNF-α when compared to other CKD patients. HD patients were then administered supplements that included 2.4g of n-3 fatty acids (180 mg EPA

and 120 mg DHA) per day for eight weeks. After treatment, there was a significant increase in DHA and EPA and a significant decrease in inflammatory markers, IL-6 and TNF-α, in the supplement group. It was concluded that nutritional factors, such as essential fatty acids, can lead to permanent changes in the inflammatory process (Perunicic-Pekovic et al., 2007). Other researchers, such as Cappelli et al. (1997), randomized 20 chronic renal failure (CRF) patients on a conservative treatment, into two different groups. Group 1 included 10 control patients and Group 2 had 10 patients that ingested a 3.4g daily dose of n-3 fatty acids, which included a mixture of EPA and DHA in the form of four soft gelatin capsules containing 1g of fish oil ethyl ester with 85% PUFA. In addition, vitamin E was added to prevent oxidation of the highly unsaturated EPA and DHA. Measurements were made in basal conditions and at the end of the 12 months. A significant decrease in IL-1β and TNF-α was found. In addition, Group 2 patients experienced a steady monthly reduction of GFR, whereas it rose progressively in Group 1(Cappelli et al., 1997). Himmelfarb et al. (2007) examined the effects of gamma tocopherol and DHA administration on inflammation and oxidative stress markers in HD patients in a randomized, double-blinded, placebo-controlled, clinical trial. Active treatment consisted of capsules containing gamma tocopherol (308 mg) and DHA (800 mg). Plasma concentrations of IL-6 were measured at the start of the study and then after eight weeks. Fifty-seven HD patients completed the study with no serious adverse events attributed to either active treatment or placebo. In the treatment group, but not in the placebo group, there was a significant decrease in IL-6 (21.4 +/- 3.5 to 16.8 +/- 3.7 pg/mL). In conclusion, gamma tocopherol and DHA were well-tolerated and reduced selected biomarkers of inflammation in HD patients (Himmelfarb et al., 2007). Yet,

continued studies are needed to determine which component, gamma tocopherol or DHA, can reduce biomarkers of inflammation and therefore reduce cardiovascular complications in HD patients.

In contrast to human model studies, animal model studies have also confirmed the benefits of n-3 fatty acids. For example, a diet was enriched with n-3 lipid rich-menhaden fish oil (FO) and fed ad libitum to autoimmune lupus-prone NZB/NZW F1 (B/W) female mice. FO delayed the onset and slowed the progression of renal disease while noticeably lengthening life-span when compared to n-6 lipid rich-corn oil (CO)-fed mice. Northern blot analysis of kidneys from FO-fed mice showed no measurable levels of IL-1 β, IL-6, and TNF-α mRNA compared to levels that were easily identified in CO-fed mice. Also, FO-fed mice showed higher renal levels of the antioxidant enzymes compared to CO-fed mice. These results suggest that dietary supplementation with FO when compared to CO, inhibits the production of pro-inflammatory cytokines and ameliorates immune-complex-mediated kidney injury by enhancing the ability of cells to dispose of harmful reactive oxygen intermediates (Chandrasekar & Fernandes, 1994).

A variety of human populations have been studied while taking n-3 supplementation, but researchers have found mixed results in inflammatory markers. For example, Tsitouras et al. (2008) investigated whether a high n-3 PUFA diet would improve inflammatory markers in six men and six women aged over 60 years and all apparently healthy. Subjects first ingested an isocaloric control diet for six weeks, followed by an 8-week experimental diet, which included 720 g of fatty fish weekly plus 15 ml of sardine oil daily. A trend towards lower IL-6 was found, but it was not significant. Meydani et al. (1991a) compared young (23-33 years) versus older women

(51-68 years) taking 1680 mg of EPA and 720 mg of DHA for 12 weeks. Both groups had a decrease in IL-1β, TNF-α, and IL-6; but only the older women had significant decreases. Esperson et al. (1992) observed 32 patients with active rheumatoid arthritis in a 12-week double-blind, randomized study of dietary supplementation everyday with either 3.6 g of n-3 PUFAs (2 g EPA, 1.2g DHA, and EPAX-5500 TG) or a placebo oil, which included a mixture of fatty acids similar to that found in the typical Danish diet (41 % saturated, 38% monosaturated, and 21% polyunsaturated fatty acids). The cytokines were measured in plasma before and after treatment in both the fish oil and placebo groups. It was concluded that dietary supplementation with n-3 PUFAs results in significantly reduced plasma IL-1 β levels in patients with rheumatoid arthritis, but there was no significant change in TNF-α activity in plasma (Espersen et al., 1992). In a double-blind, placebo controlled trial of parallel design, 59 nonsmoking, treated-hypertensive, type 2 diabetic subjects, were randomized to 4g daily of purified EPA, DHA, or olive oil for 6 weeks, while maintaining their usual diet. IL-6 and TNF-α were measured before and after intervention. Thirty-nine men and 12 women aged 61.2 +/- 1.2 years completed the intervention. There were no significant changes in IL-6 and TNF-α following EPA or DHA supplementation in the olive oil group (Mori et al., 2003).

Further, nine healthy volunteers added 18g of fish-oil concentrate (1.62g EPA and 1.08g DHA) per day to their usual Western diet for six weeks. A radioimmunoassay was used to measure IL-1 β and TNF-α produced in vitro by stimulated peripheral-blood mononuclear cells. The synthesis of IL-1 β was decreased from 7.4 +/- 0.9 ng per mL at base line to 4.2 +/- 0.5 ng per mL after six weeks of supplementation. Ten weeks after the end of n-3 supplementation, a further decrease to 2.9 +/- 0.5 ng/mL was observed and

TNF-α responded in a similar manner. Twenty weeks after the end of supplementation, the production of IL-1 β and TNF-α had returned to the pre-supplement level. The reduced production of IL-1 β and TNF-α was joined by a lower ratio of AA to EPA in the membrane phospholipids of mononuclear cells. Therefore, it was concluded that the synthesis of IL-1 β and TNF-α can be suppressed by dietary supplementation with n-3 fatty acids, contributing to its anti-inflammatory effects (Endres et al., 1989). Lastly, in a large population study that used 1123 people (aged 20-98 years), the relationship between relative concentration of fatty acids in fasting plasma and level of inflammatory markers was evaluated. After adjusting for age, sex, and major confounders, lower DHA was associated with significantly higher IL-6 and total n-3 fatty acids were associated with lower IL-6 and TNF-α. These findings support the thesis that n-3 fatty acids may be beneficial in patients affected by diseases characterized by active inflammation (Ferrucci et al., 2006).

A number of studies have investigated the effects of fish oil on the production of pro-inflammatory cytokines with most using heterogeneous blends of long-chain n-3 PUFA, EPA, and DHA. But this prevents an examination of the individual effects of n-3 PUFAs. Therefore, the differential effects of pure EPA and DHA on cytokine expression and NF-κB activation in human THP-1 monocyte-derived macrophages was evaluated. Pretreatment with 100 microM EPA and DHA significantly lowered lipopolysaccharide (LPS)-stimulated THP-1 macrophage TNF-α, IL-1β, and IL-6 production, compared to control cells. Both EPA and DHA reduced TNF-α, IL-1β, and IL-6 mRNA expression. In all cases, the effect of DHA was significantly more potent than that of EPA. Furthermore, a low dose (25 microM) of DHA had a greater inhibitory effect than that of

EPA on macrophage IL-1β and IL-6 production following 0.01 and 0.1 microg/ml LPS stimulation. Both EPA and DHA were found to down-regulate LPS-induced NF-κB /DNA binding in THP-1 macrophages by approximately 13%. Even though similar trends were seen with EPA, they were not significant. These findings propose that DHA may be more effective than EPA in decreasing LPS-induced pro-inflammatory cytokine production in macrophages, an effect that may be somewhat stimulated by NF-κB (Weldon, Mullen, Loscher, Hurley, & Roche, 2007). In another study, Vedin et al. (2008) observed the effects of n-3 fatty acid preparations with DHA as the main fatty acid. In a randomized, double-blind, placebo-controlled trial, 174 Alzheimer disease (AD) patients were given either 1.7g DHA and 0.6g EPA or placebo daily for six months. Blood samples were obtained from the first 23 randomized patients. Plasma concentrations of DHA and EPA were significantly increased after 6 months in the n-3 PUFA group. This group also showed significant decreases in IL-6 and IL-1β. Changes in the DHA and EPA concentrations were negatively associated with changes in IL-1β and IL-6 release for all subjects. Reductions of IL-1β and IL-6 were also significantly correlated with each other. Yet, this n-3 fatty acid treatment for six months did not decrease TNF-α (Vedin et al., 2008). This study reveals that AD patients treated with DHA-rich n-3 PUFAs supplementation can increase their plasma concentrations of DHA (and EPA), and reduce the associated release of IL-1β and IL-6.

Summary

In conclusion, one widespread characteristic of all stages of CKD is excessive production of inflammatory mediators, such as TNF-α, IL-1ß, and IL-6. But, potentially beneficial therapies appear to be underused and understudied in mild to moderate CKD

patients. Therefore, since n-3 fatty acid supplementation are associated with a lower level of pro-inflammatory cytokines, and consequently a decrease in severity of inflammatory diseases; increasing the intake inpatients with stages 3 and 4 of CKD through dietary supplementation could be therapeutic and possibly improve their quality of life. The benefits of n-3 supplementation for a range of populations and a variety of health concerns are evident, yet the anti-inflammatory benefits for stages 3 and 4 CKD patients are not as well documented. Therefore, continued studies in this area are clearly warranted.

CHAPTER THREE

Methods

Participants

Fifty chronic kidney disease (CKD) patients, both male and female, stages 2-5, and ≥ 18 years of age were recruited to participate in this study. Patients were recruited through Central Texas Nephrology Associates clinics under the supervision of Ronald Wilson, M.D. (Nephrologist) and through Waco Family Health Center under the supervision of Jackson Griggs, M.D. (Family Medicine). Patients were not considered for the study if they had taken an omega-3 supplement within the last three months, had allergies for fish or safflower oil (comparison group), had a current involvement in another dietary study, an active illness requiring hospitalization, their life expectancy was less than three months, had malabsorption syndromes, were pregnant, had any change in body weight (≥ 10 lbs.) in the past six months, or had a previous history of medication non-compliance. Patients age 18 and older meeting eligibility criteria were informed of the requirements of the study. All eligible participants were asked to provide oral and informed written consent based on university-approved documents and approval was granted by the Institutional Review Board for Human Subjects of Baylor University. Additionally, all experimental procedures involved in the study were conformed to the ethical considerations of the Declaration of Helsinki. The purpose of the research, the protocol followed, and the experimental procedures were explained to the participants.

Study Site

All supervised data collection was conducted at a health clinic located in Waco, Texas. Blood samples were transported from the Family Health Center to the Exercise and Biochemistry Nutrition Lab (EBNL) at Baylor University in Waco, Texas, and analyzed.

Independent and Dependent Variables

The independent variables used throughout this study included the nutritional supplementation of "Super Omega-3" and safflower oil (comparison group). The dependent variables included plasma markers of inflammation (IL-6, IL-1β, and TNF-α).

Entry/Familiarization Session

Participants that expressed interest in participating in the study were interviewed on the phone and/or in person by AmeriCorps volunteers to determine whether they appeared to qualify to participate in the study. Participants believed to meet eligibility criteria were then invited to attend an entry/familiarization session. Once reporting to the clinic, participants completed a medical history questionnaire and underwent a general physical examination by a physician to determine whether they met eligibility criteria. Participants meeting entry criteria were familiarized to the study protocol by way of a verbal and written explanation outlining the study design. At the conclusion of the entry/familiarization session, each participant was given an appointment time to begin the study.

Experimental Protocol

This experimental protocol was based on the protocol established by Schmitz et al. (2002) and is presented in Table 1. Subjects were familiarized to the experimental procedures and signed informed consent statements in compliance with the Baylor University and *Central Texas Nephrology Internal* Review Board for the Protection of Human Subjects in Research and *Providence Hospital, Waco Texas, Institutional Review Board*. Prior to study initiation, a full physical and cardiovascular examination was performed by the treating physician. The participants' diets were not standardized and subjects were asked not to change their dietary habits during the course of the study. Participants were randomly chosen to receive either "Super Omega-3" or comparison supplement (safflower oil). Randomization was configured by a website (www.random.org) that randomly assigned the participant numbers to either the treatment or placebo group. Participants were required to attend three testing sessions. At each testing session, participants had their height, weight, blood pressure, and heart rate measured. In addition, a side-effects questionnaire was completed by each participant. Participants then returned to the lab the next morning, at least eight hours fasted, to donate approximately 50 milliliters of blood, per physician's request. This was used for later analysis of plasma inflammatory markers at the first testing session. After the initial blood draw, participants were given a two-month supply of either "Super Omega-3" or placebo. Participants were instructed to ingest two capsules twice day for 28 days with meals. Following eight weeks of supplementation, participants again returned to the clinic for the same battery of measurements that were performed at the first testing session.

Table 1

Overview of Research Design

T1 - Week 0	T2 - Week 8 (56 days)
Informed Consent	Fasting Blood Samples Obtained
Personal History/Medical History Form	Return Pill Bottles
Meet with Physician to determine if qualifications allow to participate in the study	BP, HR, Weight
BP, HR, Weight, Height	Side Effects Questionnaire
Side Effects Questionnaire	Meet with physician (if needed)
Schedule appointments for blood draws (0 and 8 weeks)	
Randomized, double-blind assignment to consume either Fish oil or comparison supplement	
Fasting Blood Samples Obtained	

Supplementation compliance was monitored by having the subjects return supplement bottles with any remaining pills at the end of eight weeks of supplementation. The standard practice of pill counting, counting the remaining pills, and calculating the percentage left, was also used to assess compliance. Subjects were also called by the investigator periodically to check for any side-effects and for compliance. It has been recommended by some study authors that the standard for compliance should be between 80-100% (Park et al., 2005). Therefore, patients that consumed 80% of the issued supplements in this study were considered compliant.

"Super Omega-3" (Fish Oil) and Comparison Supplement Composition

"Super Omega-3" and safflower oil capsules are quality assured and quality controlled by *Life Extension* (Ft. Lauderdale, FL) (See Appendix F). One capsule of "Super Omega-3" contains 350 mg of EPA, 250 mg of DHA, 150 mg of Olive Fruit Extract, and 5 mg of Sesame Ligan Extract. For full benefits, it is suggested to take four capsules of "Super Omega-3", for a total of 1400 mg of EPA, 1000mg of DHA, 600 mg of Olive Fruit Extract, and 20 mg of Sesame Ligan Extract per day. Therefore, patients consumed a total of 2.4 g of omega-3 fatty acids (EPA + DHA). The comparison supplement was composed of safflower oil, high in unsaturated omega-6 fatty acids. Safflower oil has been used as the placebo in other n-3 fatty acids studies and has not been shown to significantly decrease cytokines in humans when compared to n-3 fatty acids (Ciubotaru, Lee, & Wander, 2003; Rallidis et al., 2003).

Assessment of Body Mass and Height Measurements

Body mass and height was measured at baseline and body mass was measured again at eight (8) weeks. Measurements were taken by a doctoral student or a trained volunteer on a medical scale accurate to ±0.02 kg. The scale also had a standard measuring rod for measuring height.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure)

Participants underwent the assessment of hemodynamic safety markers (heart rate and blood pressure) at each testing session. Heart rate and blood pressure was assessed in the seated position using a digital blood pressure monitor.

Reported Side Effects from Supplements Questionnaire

At the conclusion of each of the two testing sessions, participants reported by questionnaire whether they tolerated each supplement. In addition, participants were told to report any medical problems/symptoms they may have encountered throughout the duration of the study through weekly phone calls. However, if symptoms/complications were to arise prior to completing the questionnaire, participants were encouraged to report them as they occurred.

Blood Collection

Participants donated approximately 50 milliliters of fasting venous blood at the beginning of the study and then after eight weeks of supplement use. Blood was taken into 5 ml plasma separation vacutainer tubes (heparin-green tops). Blood samples were obtained after a 12-hour fast and standardized to the same time of day for each sample, meaning participants gave blood samples at the same time of day for pre and post blood draws. Blood samples were obtained by inserting a needle into an antecubital vein using standard phlebotomy procedures by nurses at the clinic that are trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. Nurses wore personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Blood samples were allowed to stand at room temperature for 10 minutes and then centrifuged. The blood collection tubes were labeled and placed in a test tube rack. Laboratory technicians (had received blood borne pathogen training and wore personal protective clothing) centrifuged the serum samples at 2,400 rpm for 10 minutes. Blood was then transported in an insulated cooler to the EBNL. Once at the

EBNL, the plasma was transferred into labeled storage containers, and stored at -20°C for later analysis.

Analysis of Plasma Markers of Inflammation

Plasma levels of IL-1β, IL-6, and TNF-α was analyzed using commercially available enzyme immunoassay [(EIA), Cayman Chemical, Ann Arbor, MI]. The immunometric assay is based on a double-antibody 'sandwich' technique. First, 100 µl of standards and samples will be placed into their corresponding well. Following this step, 100 µl of the acetylcholinesterase:Fab' Conjugate was added to each well except the blank. The plate was then covered and sealed and incubated overnight at 4ºC. Following the overnight incubation, the wells were emptied and rinsed five times with Wash Buffer. Following the wash steps, 200 µl of Ellman's reagent was added to all wells. The plate was covered with plastic film and placed on the plate shaker at room temperature in the dark for approximately 30-60 minutes. Once proper color (yellow) development was made, the plate was immediately read at a wavelength between 405 and 420 nm using the Wallac Vicotor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). According the kit insert, the intra-assay coefficient of variation for this assay was <25% and the inter-assay coefficient of variation was <30% (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

The Statistical Package for the Social Sciences software for Windows (SPSS© Inc, Version 18.0, Chicago, IL) was used to perform the statistical analysis of the data. The primary outcome variables were IL-6, IL-1β, and TNF-

α. Separate repeated measures analysis of variance (ANOVA) was used to measure changes in primary outcome variables using a 2 (fish oil or safflower oil) x 2 (time points) design. Significance level was set at $p \leq 0.05$.

CHAPTER FOUR

Results

Demographics

From the 53 individuals that volunteered to participate for this study, only 31 completed the study. Self-reported reasons for not completing the study included: the pills being difficult to ingest, forgetting to take the supplement, or an unrelated illness, such as pneumonia. In addition, several patients' phones were disconnected, leaving no possibility of contacting them for continuance in the study. Sixteen males and 15 females (Table 2) completed eight weeks of either the fish oil or safflower oil (comparison group) supplementation for a 58% completion rate**.** The fish oil (FO) group concluded with 17 individuals and the safflower oil group included 14 individuals. Based on the representation of ethnic group in the FO and safflower oil groups, the makeup of the subjects included African Americans, Caucasians, and Hispanics, and the groups were not found to be significantly different ($p = 0.64$). Further, this study required patients to have between stage 2 and 5 CKD. All demographic information is presented in Table 2. At baseline, age, height, weight, and waist circumference were measured and the groups were compared with no significant difference in age ($p=0.94$) or height ($p=0.35$). FO group displayed significantly higher body weight ($p=0.04$) and waist circumference ($p=0.02$) compared to the safflower group.

Table 2

Descriptive Statistics for Fish Oil (FO) and Safflower Oil (SO) Groups

Variable	FO Group n = 17	SO Group n = 14	*p* Value
Sex, n (%)			0.93
Male	8 (35.29)	9 (48.57)	
Female	9 (64.71)	5 (51.43)	
Ethnicity, n (%)			0.64
African-American	11 (64.7)	10 (71.4)	
Caucasian	4 (23.5)	3 (21.4)	
Hispanic	2 (11.8)	1 (7.2)	
State of CKD			0.18
2	2	1	
3	12	9	
4	3	3	
5	0	1	
Average Age, y±SD	64±10	62±10	0.94
Average Height (cm)	174±11	169±9	0.35
Average Weight (kg)	108±28	98±23	0.04
Average Waist (cm)	119±25	108±15	0.02
Average SBP (mmHg) ± SD	135±25	137±17	0.02
Average DBP	73±14	77±17	0.39
Average Heart Rate	78±11	75±15	0.35

Note: CKD: Chronic Kidney Disease FO: Fish Oil
SBP: Systolic Blood Pressure DBP: Diastolic Blood Pressure

Supplement Compliance

At the start of the study, participants were instructed to take their supplement in the morning and evening, everyday for eight weeks. The FO group ingested two capsules in the morning and two in the evening, while the comparison group was instructed to take one in the morning and one in the evening. Participants were given their supplements and instructions by the pharmacy, located at a health center in Waco, TX, for a double blind study. The pharmacy was provided instructions from the physicians working with this current study. It was emphasized to the participants that it could affect the outcome of the study if they did not take all of their supplements in which they were supplied. But, they were also told that if the supplement was not taken for any reason, they would not be denied supplements or the ability to finish the study. Participants were just asked to report how often they missed taking their required dosage. Throughout the eight week protocol, participants were called weekly to verify that they were taking their supplements on a regular basis. To establish how many capsules were not taken, participants were asked to bring their supplement bottles to their final testing session (T2), where remaining pills were counted to determine compliance. The average compliance rate was 82%, with a range from 43% to 100%. In the FO group, 10 out of the 17 were compliant, with a 59% compliance rate. For the comparison group, 8 out of the 14 were compliant, with a 57% compliance rate. When a statistical analysis was run for those that were 80% or more compliant, the results were still not significant. Reasons for subjects not being compliant included forgot to take the supplement, not feeling well, or elected not to take the supplement.

Side Effects

All participants filled out a side effects questionnaire at the beginning of the study (T1) and then at the conclusion of the study (T2). In addition, participants were asked in their regular scheduled phone calls whether they were experiencing any adverse side effects. The most common side effect was a foul taste in their mouth; others listed were upset stomach, a change in bowel habits, fast or racing heart rate, dry mouth, dizziness, and a decreased appetite. Previous research has reported increased burping, indigestion, abdominal bloating, and abdominal discomfort as being common with the use of fish oil supplements. It has been found that gastrointestinal side effects can be reduced if fish oils are taken with meals and if doses are started low and gradually increased. Another common side effect listed is a fishy after-taste (Berbert, Kondo, Almendra, Matsuo, & Dichi, 2005; Cappelli et al., 1997; Fiedler et al., 2005; Saifullah et al., 2007).

Markers of Inflammation

The independent variables used throughout this study included the nutritional supplementation of fish oil and safflower oil. The dependent variables included plasma markers of inflammation (IL-6, IL-1β, and TNF-α). Repeated Measures ANOVA was used to measure changes in the primary outcome variables using a 2 (fish oil or safflower oil) x 2 (time points) design. Significance level was determined a priori at $p < 0.05$.

TNF- α

In evaluating TNF- α levels, there was no significant difference between groups ($p = 0.23$) at the start of the study (T1). The averages were determined for the FO group at T1 (16 ±15.4 pg/mL) and T2 (23 ±19.7 pg/mL) and the safflower oil group at T1

(22.5±11 pg/mL) and T2 (16 ±7.2 pg/mL) (Figure 4). The analysis of TNF-α levels revealed no significant difference across time (p = 0.92) and between groups (p = 0.94), yet there was a However, there was a group by time interaction, indicating that the average TNF-α level changed over time differently between groups (p = 0.03). While TNF-α levels in the FO group went up, the placebo group went down.

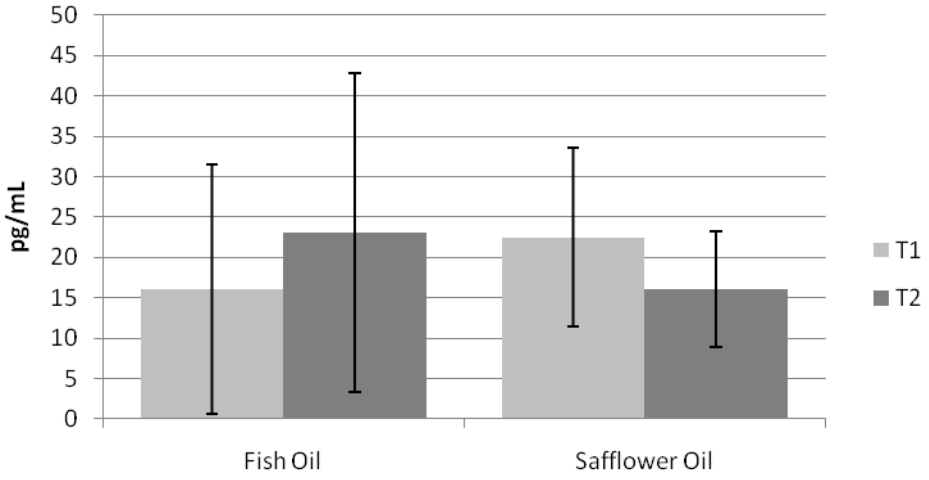

Figure 4. Means and standard deviations of TNF-α in pg/mL at T1 and T2 in subjects taking fish oil and safflower oil. The analysis of TNF-α levels revealed no significant difference across time (p = 0.92) or between groups (p = 0.94). Group by time interaction was significant (p = 0.03).

IL-6

In evaluating Il-6 levels, there was no significant difference between groups (p = 0.58) at the initial testing session (T1). The averages as determined for the FO group at T1 (10 ±6.60 pg/mL) and T2 (14 ± 8.30 pg/mL), and for the safflower oil group at T1 (13 ±13.20 pg/mL) and T2 (11.6 ± 16 pg/mL) (Figure 5). The analysis between groups and IL-6 levels resulted in no significant difference across time (p = 0.30) or between groups (p = 0.15), nor was there a significant group by time interaction with the trends across

time differing by group membership (p = 0.82). Yet, there was a trend similar to that found in TNF- α, where IL-6 levels in the FO group went up and the safflower oil group went down.

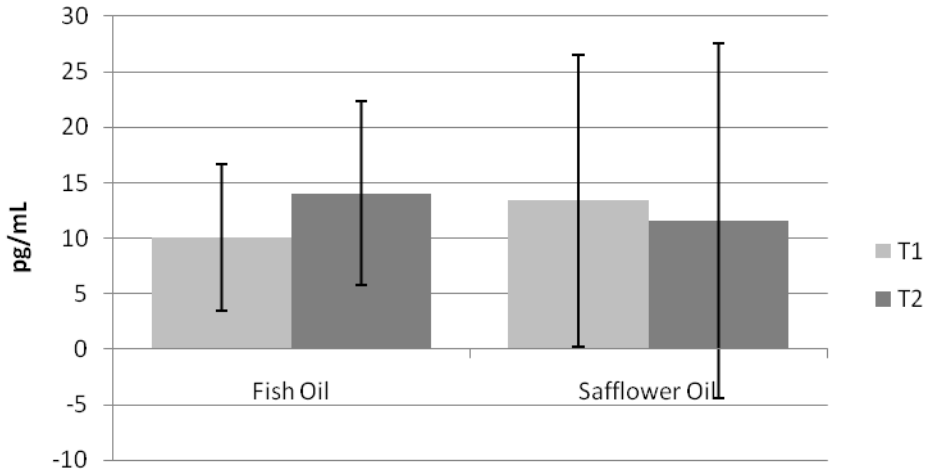

Figure 5. Means and standard deviations of IL-6 (pg/mL) at T1 and T2 in subjects taking fish oil and safflower oil. The analysis resulted in no significant difference across time (p = 0.30), between groups (p = 0.15), or group by time interaction (p = 0.82).

IL-1β

When assessing IL-1β levels, no significant difference between groups (p=0.09) at the initial testing session (T1). The averages for each group were calculated for FO group T1 (10.7 ±6.5 pg/mL), T2 (14 ± 7.9 pg/mL), and for the safflower oil group T1 (12 ±6.5 pg/mL) and T2 (8.1 ± 8.5 pg/mL) (Figure 6). The analysis between group and IL-1β resulted in no significant difference in IL-1β levels across time (p=0.17) or between groups (p=0.26); moreover, there was no group by time interaction (p=0.44). Again, there was a trend similar to TNF- α and IL-6, where IL-1β levels in the FO group went up and levels in the safflower oil group went down.

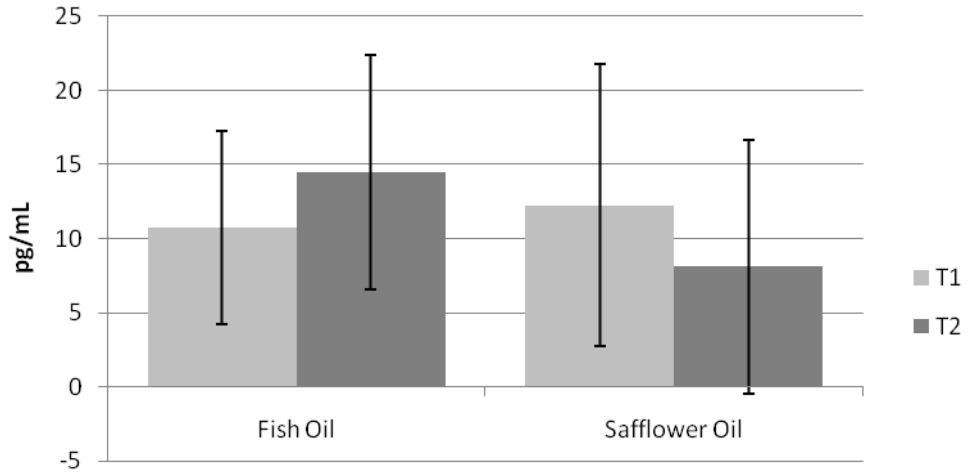

Figure 6. Means and standard deviations of IL-6 in pg/mL at T1 and T2 in subjects taking fish oil and safflower oil. The analysis resulted in no significant difference in IL-1β levels across time (p=0.17), between groups (p=0.26) or group by time interaction (p=0.44).

CHAPTER FIVE

Discussion

Inflammation is a multifaceted process that is associated with an abundance of chronic conditions and diseases. Therefore, the more that is known about what influences inflammation, the better understood how to alter disease risk. In the current study, the focus was on CKD patients who normally have a high degree of inflammation (Madsen et al., 2003; Rasic-Milutinovic et al., 2007; Saifullah et al., 2007). Previous research has shown in other populations that supplementing the diet with fish Oil (FO) can decrease inflammation, confirmed by a decrease in pro-inflammatory cytokines (De Caterina, Endres, Kristensen, & Schmidt, 1994; Perunicic-Pekovic et al., 2007). But, evidence regarding the causative role of FO is inconclusive. Further, there are only a small number of studies that have been conducted in CKD patients. These few studies have only focused on dialysis patients, not patients with stages 2-5 of CKD. For that reason, our study included CKD patients, not on dialysis.

The current study examined the effects of fish oil (FO) supplementation on pro-inflammatory markers TNF-α, IL-6, and IL-1 β on CKD patients, stages 2-5 after eight weeks of supplementation. Each FO supplement contained 2.4 g of omega 3 polyunsaturated fatty acids (n-3 PUFAs), including 1400 mg EPA + 1000 mg DHA. It was hypothesized that a supplementation of FO, a source of n-3 PUFAs, would promote a decrease in systemic inflammation and, therefore, a decrease in TNF-α, IL-6, and IL-1 β. This hypothesis was based on the findings of many previous studies that used supplementation with n-3 PUFAs and revealed anti-inflammatory effects in animals and

patients with other chronic conditions; therefore, it could theoretically be of significance in patients with renal diseases (De Caterina et al., 1994; Perunicic-Pekovic et al., 2007). Therefore, since TNF-α, IL-6, and IL-1 β did not decrease when using a FO supplement, the study hypotheses were rejected.

One possible explanation for the lack of alterations in the markers of inflammation could be because of the duration of treatment in the current study. Previous studies supplemented FO for six months to a year (De Caterina et al., 1994) suggesting eight weeks was not long enough to produce more favorable effects.

Additionally, the study population presented a number of challenges with the current study. The patients had a number of co-morbidities and consequently were already taking a number of medications which may have caused pill fatigue and decreased compliance. Patients also complained about how large the pills were and how difficult it was to ingest the supplement. Further, two patients developed pneumonia and required hospitalization. Lastly, problems were encountered when communicating with study participants. Phone calls reminding patients to take their pills and to come to their schedule appointment was a challenge. Many had their phones disconnected or did not have answering machines. Yet, when contacted several individuals stated they had no transportation to get to their appointment. Also, instructions were repeated multiple times to make sure each participant understood the protocol, but issues still arose, such as going to the wrong location, not fasting, and thinking they could buy their own FO supplement while traveling. In addition, many did not have computers, making e-mail communications not possible.

Inflammation and Fish Oil

Several former studies have suggested that n-3 PUFAs can exert favorable effects in patients with CKD, but the mechanisms involved are not yet fully understood (Perunicic-Pekovic et al., 2007; Zhao et al., 2009). With numerous study authors reporting equivocal results the evidence for its benefit is still needs further study, (Cappelli et al., 1997; De Caterina et al., 1994; Espersen et al., 1992; Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007; Szklarek-Kubicka et al., 2009). Mounting evidence has emerged that FO-derived n-3 PUFAs provide cardio-protective benefits by decreasing inflammation, (Cappelli et al., 1997; De Caterina et al., 1994; Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007), but our study did not support these findings.

Differences with other studies might be explained by limitations of the current study's design, the dose of FO used (Endres et al., 1989), by the differences in their diet (Meydani, Endres et al., 1991b) or elevated inflammation levels prior to the start of the study (Blok et al., 1997; Saifullah et al., 2007). In studies conducted with healthy populations, such as those without any known underlying disease such as diabetes, cancer, or coronary heart disease, low concentrations of circulating cytokines are often found. Therefore, this makes the benefits of FO harder to detect. Yet, studies using individuals in severe disease states with extremely high baseline cytokine levels, such as septic shock, burn trauma, or dialysis patients, the benefits of FO are more often observed (Blok et al., 1997; Saifullah et al., 2007). For example, Pot et al. (2009) conducted a study to determine the effects of FO supplementation on 19 serum inflammatory markers, including TNF-α, IL-6, and IL-1 β, and their interrelationships in 77 healthy individuals,

aged 50-70 years, in a randomized, double-blind placebo-controlled intervention study. Participants received 3.5 g/day FO (1.5g/day n-3 PUFA) (n = 39) or placebo (safflower) (n = 38) for 12 weeks. FO supplementation did not significantly affect any of the serum concentrations of pro-inflammatory cytokines when compared with placebo. If anything, there was a trend of all serum inflammatory markers increasing after FO supplementation, but the changes were not statistically significant, which is similar to the current study. Pot et al. (2009) concluded that since healthy individuals usually have low levels of inflammatory markers, the chance that low levels of inflammation will be reduced with a FO supplement is very unlikely. Further, another explanation was that blood samples were collected throughout the day, which could have caused some differences due to natural fluctuations (Pot et al., 2009). In the current study, attempts were made to have patients come at the same time to donate blood while fasted. Luu et al. (2007) investigated how n-3 PUFAs modified the ability of monocytes to conduct an inflammatory response, which is one of their key functions. It was found that FO supplementation decreased the strength of monocytes in normal subjects, but not those from patients with peripheral artery disease (PAD). It was concluded that medication taken simultaneously may have acted as a mitigating factor because those receiving medication might have a more activated phenotype that was less responsive to n-3 PUFAs (Luu et al., 2007). This is another possible reason why we saw no decreases in pro-inflammatory markers in our current study. In addition, Saifullah et al. (2007) found large reductions in saturated and monounsaturated fatty acids seen in erythrocytes as a result of 12 weeks of n-3 PUFA supplementation, but not in plasma levels, which is most likely due to preferential uptake of n-3 PUFAs (Saifullah et al., 2007). Thus, it is

possible that other markers of immune function, such as the expression of cell adhesion markers on monocytes and respiratory burst response in neutrophils, could have been influenced by changes in inflammation (Luu et al., 2007; Rees et al., 2006).

TNF-α

The present study evaluated changes in the pro-inflammatory cytokine, TNF-α. TNF-α is closely associated to inflammation and is the first cytokine that has an indirect role in promoting insulin resistance by increasing the release of free fatty acids from adipose tissue (Calder, 2007; Park et al., 2005). But previous studies have reported that TNF-α could possibly be decreased with FO supplementation (Cappelli et al., 1997; Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007). Therefore, it was hypothesized that there would be a decrease in plasma levels of TNF-α following eight weeks of FO supplementation in CKD patients. This hypothesis was based on the findings of previous studies that reported decreased TNF-α levels when using FO supplementation in CKD patients (Cappelli et al., 1997; Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007) as well as other populations (Endres et al., 1989; Meydani et al., 1991b; Sundrarjun et al., 2004; Zhao et al., 2009). For example, Perunicic-Pekovic et al. (2007) observed 42 hemodialysis (HD) patients (mean age 55 +/- 8 years) and compared them to a control group that consisted of 16 healthy subjects of similar age and sex to the tested group. HD patients were administered supplements with 2.4 g of n-3 PUFAs per day for eight weeks. At the start of the study, subjects were found to have elevated plasma TNF-α levels. But, after eight weeks of treatment, a significant decrease in the inflammatory marker TNF-α was found. Yet, this study lacked a placebo group, therefore, challenging the idea of its significance (Perunicic-Pekovic et al., 2007). Zhao

et al. (2009) found that treatment with three months administration of n-3 PUFAs at 2 g/day, significantly decreased plasma levels of TNF-α in patients with heart failure. The study authors reasoned that n-3 PUFAs decreased inflammation because they are precursors of anti-inflammatory eicosanoids (such as prostaglandin I3, prostaglandin E3, thromboxane A3, and leukotriene B5), and consequently are believed to attenuate the inflammatory response. It was also suggested that n-3 PUFAs can join peroxisome proliferator activated receptors (PPAR) α and γ, which can inhibit gene transcription by interfering with signaling molecules, such as factor (NF)-κB, thereby blocking the formation of pro-inflammatory cytokines, chemokines, and adhesion molecules (Zhao et al., 2009). Further, when nine healthy volunteers added 18 g of FO concentrate per day to their usual Western diet for six weeks, TNF-α was found to be suppressed. The decreased production of TNF-α was joined by a decreased ratio of AA to EPA in the membrane phospholipids of mononuclear cells. They reported the reason for the anti-inflammatory effects of these n-3 PUFAs may be mediated at least partly by their inhibitory effect on the production of TNF-α (Endres et al., 1989).

However, the current study does not agree with the literature. The averages for this study were determined for the FO group at T1 (16 ±15.4 pg/mL) and T2 (23 ±19.7 pg/mL) and the safflower oil group at T1 (22.5±11 pg/mL) and T2 (16 ±7.2 pg/mL). These levels were lower than some studies that observed dialysis patients (Espinoza, M., et al., 1999). A trend was seen with the fish oil group increasing TNF- α levels, while the safflower oil group decreased these levels. But, due to the fact that TNF- α did not decrease when using a FO supplement, the hypothesis stating that there would be a decrease in plasma levels of TNF-α following eight weeks of FO supplementation in

stages 2-5 of CKD patients was rejected. A possible explanation for the lack of change could be because TNF- α works locally, such as within the cell, rather than throughout the body. Therefore, TNF-α transcription may not always be reflected in enhanced systemic levels of TNF- α. In addition, differences might be explained by study design, the dose of FO used or higher levels of inflammation prior to the study, such as in dialysis patients (Endres et al., 1989). One study found similar results to this one in that they concluded that dietary supplementation with FO does not significantly reduce plasma TNF-α levels in patients with rheumatoid arthritis (Espersen et al., 1992).

IL-6

Another role of this study was to investigate the changes in the pro-inflammatory cytokine IL-6. IL-6 is thought to be released in direct response to TNF-α and there may be a linear correlation between these two pro-inflammatory cytokines (Zhao et al., 2009). Previous research reported that IL-6 is a central mediator of cardiovascular risk, associated with many diverse conditions, and that the effects of FO supplementation on IL-6 levels are still not well defined (McCarty, 1999). Therefore, it was hypothesized that a significant decrease in IL-6 levels would occur. This hypothesis was again based on the findings of past studies that concluded that short-term administration of FO would decrease the pro-inflammatory cytokine IL-6 in CKD patients (Cappelli et al., 1997; Himmelfarb et al., 2007; Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007) and in other populations (Ferrucci et al., 2006; Meydani et al.1991b; Sundrarjun et al., 2004).

Ferrucci et al. found that plasma PUFA levels were independently associated with lower levels of inflammatory markers IL-6 and higher levels of anti-inflammatory marker

(soluble IL-6 receptor) (Ferrucci et al., 2006). Zhao et al. (2009) concluded that treatment with 12 weeks administration of n-3 PUFAs at 2g/day, significantly decreased plasma levels of IL-6 in patients with heart failure. Study authors reasoned that n-3 PUFAs decreased inflammation because they are precursors of anti-inflammatory eicosanoids and therefore believed to attenuate the inflammatory response (Zhao et al., 2009). Perunicic-Pekovic et al. (2007) observed 42 HD patients and evaluated them against a control group that included 16 healthy subjects of similar age and sex to the tested group. HD patients were administered supplements with 2.4 g of n-3 PUFAs per day for two months. At the initial start of the study, subjects were found to have increased plasma IL-6 levels. But, after two months of treatment, a significant decrease in inflammatory marker IL-6 was found (Perunicic-Pekovic et al., 2007).

In contrast, with the current study FO supplementation did not cause a decrease in IL- 6 levels. The averages for the current study were determined for the FO group at T1 (10 ±6.60 pg/mL) and T2 (14 ± 8.30 pg/mL), and for the safflower oil group at T1 (13 ±13.20 pg/mL) and T2 (11.6 ± 16 pg/mL). Due to the fact that IL-6 did not decrease when using a FO supplement, the hypothesis stating that there would be a decrease in plasma levels of IL-6 following eight weeks of fish oil supplementation in stages 2-5 of CKD patients was rejected.

There are studies that concur with our current results such as a study conducted by Szklarek-Kubicka et al. (2009). They evaluated 20 HD patients that received 100 mL of 10% n-3 PUFA emulsion during 11 consecutive HD sessions. After four weeks, no significant changes in IL-6 levels were found. They concluded that short-term parenteral administration of FO supplementation does not significantly influence markers of

inflammation in chronic HD patients, but suggested it may attenuate the inflammatory response in the HD sessions (Szklarek-Kubicka et al., 2009).

IL-1β

A final purpose of our study was to investigate the changes in IL-1β. We hypothesized that there would be a decrease in plasma levels of IL-1β levels following eight weeks of FO supplementation in CKD patients. This hypothesis was based on the findings of previous studies that reported decreased IL-1β levels when using a short term administration of FO in CKD patients (Cappelli et al., 1997; Endres et al., 1989; Espersen et al., 1992), and in other populations (Sundrarjun et al., 2004). For example, one study concluded that dietary supplementation with FO results in significantly reduced plasma IL-1 β levels in patients with rheumatoid arthritis. Espersen et al. (1992) stated that a possible explanation for why there was a decrease in IL-1 β was because of n-3 PUFAs causing a down regulation of prostaglandin E-2 following IL-1 β production by monocytes. Yet, the patients used NSAIDs, which is known to block prostaglandin production. In that case, the decrease in IL-1β plasma levels could have been in part the result of decreased leukotreine B4 synthesis and generation of FO derived leukotriene B5 (Espersen et al., 1992).

In the current study, the averages for each group were calculated for FO group T1 (10.7 ±6.5 pg/mL), T2 (14 ± 7.9 pg/mL) and for the placebo group T1 (12 ±6.5 pg/mL) and T2 (8.1 ± 8.5 pg/mL). The results showed that IL-1β did not decrease over time, which supports previous studies. Due to these findings, the hypothesis stating that there would be a decrease in plasma levels of IL-1 β following eight weeks of fish oil supplementation in stages 2-5 of CKD patients was rejected.

For example, a long-term placebo-controlled study with 58 monks as participants, with a mean age of 56 years, were randomized into four groups and their diets were supplemented with either 0, 3, 6, or 9g of FO, providing 0, 1, 2, or 3g of n-3 PUFAs per day. Whole-blood cytokine production was measured at 26 weeks and 52 weeks after the start of the study and then 4, 8, and 26 weeks after discontinuation of the supplement. In all groups, IL-1 β was actually higher during supplementation, compared to when the supplement was discontinued. This study concluded by stating that long-term supplementation of FO does not decrease ex vivo cytokine production during supplementation, or after cessation of supplement when compared to placebo. They reasoned that statistical testing of changes from baseline within the FO group in other studies could have lead to the inaccurate conclusion that FO does decrease cytokine levels of production in previous studies. Further, they hypothesized that the effects of n-3 PUFAs that are apparent at six weeks may have disappeared at 26 weeks of dietary FO supplementation. Blok et al. (1997) also found that in their subjects that the alterations in fatty acid profiles were comparable to those in other studies mentioned that had found a decrease in inflammation markers. Therefore, it was reported that the dose of n-3 PUFAs used did not account for the lack of an effect on cytokine production (Blok et al., 1997). Yet, the amount of inflammation the subject had at the start of the study is a significant factor in the effects on IL-1β (Blok et al., 1997; Saifullah et al., 2007).

Conclusions

In conclusion, the results of this study is in agreement with some previous studies that suggest that FO supplementation does not significantly decrease plasma pro-inflammatory markers TNF-α, IL-6, and IL-1 β in CKD patients. But, there was a trend

seen with the pro-inflammatory markers going up in the FO group and then markers going down in the comparison group. Further investigation is required to better define the long-term impact of FO supplementation in this high-risk population.

There were a number of limitations to the present study. First, the relatively small sample size and no follow-up period restricted statistical power (which could have prevented us from detecting statistically significant differences between groups) and limited our long-term observations on compliance, adverse effects, and hard clinical outcomes. In addition, this study had a low compliance rate, with the average compliance rate of 82%, ranging from 43% to 100%. Reasons for subjects not being compliant included, forgetting to take the supplement or they were just not feeling well and decided not to take it. This factor had a huge impact on our current study in that it is difficult to make a conclusion when the full amount of supplement is not taken.

These findings may help future researchers to identify new strategies to determine how inflammation can be decreased in patients with CKD, hypertension, heart disease, diabetes, etc. Future studies could conduct a thorough nutritional interview of CKD patients, which could provide help in planning treatment strategies aimed at improvements in nutritional status. We did not find the intervention to decrease markers of inflammation, but we did demonstrate that a short-term administration of FO is well-tolerated by CKD patients.

APPENDICES

APPENDIX A

IRB Proposal

Application to the Baylor IRB
For Review of Research/Activity Proposal
Part 1: Signature Page

1. Name Dr. Rodney Bowden

2. Email address (optional) Rodney_Bowden@baylor.edu

3. Complete Mailing Address P.O. Box 97313, Waco, TX 96798-7313

4. Position PhD Candidate

5. Faculty Advisor (if researcher is Graduate Student) _____

6. Dept./School Health, Human Performance, and Recreation/School of Education

7. Telephone Number 254-710-3241 Fax Number 254-710-3527

8. Are you using subjects in research (Y or N) Y or in teaching exercises? (Y or N) N

9. Title of the research project/teaching exercise: Effects of Omega-3 Fatty Acids on Inflammatory Markers in Chronic Kidney Disease Patients

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; Dr. Matt Stanford, Chairman, Department of Psychology and Neuroscience, Baylor University, P.O. Box 97334, Waco, Texas 76798-7334. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Stanford at extension 2961.

_____ _____
Signature of Principle Investigator Date

_____
Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review _____
Department Chair or the Chair's Designate

Part 2: Introduction and Rationale

Many chronic diseases are associated with low-grade systemic inflammation evidenced by a two to threefold increase in pro-inflammatory cytokines during the disease process. Cytokines are regulatory proteins which are released by cells of the immune system and act as intercellular mediators in the generation of an immune response. Examples of pro-inflammatory cytokines include: tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and interleukin (IL)-1β. An increased release of pro-inflammatory cytokines, such as IL-6, TNF-α or IL-1β, may cause anorexia, muscle proteolysis and hypoalbuminemia, and most likely contributes to atherogenesis (1-3). Further, adipose tissue-derived TNF-α has been suggested to play a direct role in the metabolic syndrome (1-3). Serum C-reactive protein (CRP) is an acute phase protein produced by the liver and adipocytes and is also an indicator of inflammation. CRP levels have been reported to be a stronger predictor of cardiovascular events comparable to LDL levels as they are an important marker of vascular inflammation and atherosclerosis (1-3).

Study authors have reported that the use of omega-3 (n-3) fatty acids, found in fish oil, lowers systemic inflammation by decreasing inflammatory markers, such as TNF-α, IL-6, and IL-1β (4). Experimental and in vivo studies have shown n-3 fatty acids to have anti-inflammatory, antithrombotic, and antiarrythmic properties and have been found to improve insulin sensitivity and lipid profiles. In addition, data has suggested that dietary fish oil is inversely correlated with CRP levels. In contrast, an abundance of omega-6 (n-6) fatty acids in the diet are known to increase inflammation, thrombosis and insulin resistance (5). It is postulated that the benefits of n-3 fatty acids are found in its

role in reaching a balance with n-6 fatty acids (6). Study authors (6, 7) have concluded that the n-6/n-3 ratio should be about 1-4/1 rather than the ratio of 20-16/1, which is currently the makeup of the Western diet. An increase in the consumption of dietary n-6 fatty acids, such as Linoleic Acid (LA) and Arachidonic Acid (AA), leads to oxidation of low-density lipoprotein (LDL), platelet aggregation, and interferes with the inclusion of n-3 fatty acids in the cell membrane of phospholipids, and thereby increasing inflammation (8). Subsequently when diets are supplemented with n-3 fatty acids they can replace the n-6 fatty acids in the membranes of practically all cells, such as erythrocytes, platelets and endothelial cells, exerting anti-inflammatory effects. This decrease in inflammation is concomitant with a reduction in both serum cytokine concentrations and in the production of pro-inflammatory cytokines (8, 9).

A population that is impacted by the effects of inflammation includes Chronic Kidney Disease (CKD) patients. They experience a multitude of conditions that are associated with the development of systemic inflammation, which can negatively affect their quality and length of life. Data suggests, (5) that a low level of n-3 PUFAs in the blood of CKD patients may contribute to the many inflammatory complications, including dialysis access thrombosis and cardiovascular outcomes. Additionally, the American Heart Association recommends that patients with high risk for cardiovascular morbidity and mortality, such as CKD patients, consume at least 1g of fish oil daily as well as maintain a dietary ratio of 4:1 for omega-6 to omega-3 (1, 3).

CKD severity is classified in stages. Patients with stage 1 CKD have normal glomerular filtration rate (GFR) yet with abnormalities such as proteinuria. More advanced losses of GFR are classified Stages 2 (estimated GFR 60-90 cc/min), stage 3

(estimated GFR 30-60 cc/min), stage 4 (estimated GFR 15-30 cc/min), to stage 5 (with less than 15 cc/min) with or without dialysis therapy. The few studies that have been conducted on n-3 fatty acids, inflammatory markers and CKD patients have included ESRD or stage 5 CKD patients. These studies have reported equivocal results. Positive results were found by a study that reported a significant decrease in IL-6 and TNF-α, after eight weeks of supplementing 2.4g/day of n-3 (180 mg of EPA and 210 mg of DHA) (10). Saifullah et al., 2007 reported a 24% decrease in CRP levels in patients who consumed 1.3 gram of n-3 (containing 427 mg of EPA and 244 mg of DHA) per day (11). Likewise, Madsen et al found decreasing CRP levels after supplementation of n-3 (containing 300 mg of EPA and 210 mg of DHA) for eight weeks (12). Cappelli et al. found a decrease in IL-1β, IL-6, and TNF-α after 12 months in CKD patients ingesting 3.4g n-3 (13). In addition, study authors supplementing patient diets with n-3 in other populations such a rheumatoid arthritis and healthy populations have also reported positive results in decreasing these inflammatory markers. The amount of n-3 in these studies ranged from 1.3g to 2.4g with the level of EPA and DHA ranging from 180mg to 427mg and 120mg to 244mg, respectively (14-19).

In contrast, one study of ESRD patients that supplemented n-3 fatty acids for 8 weeks did not observe significant changes in serum CRP concentrations when patients consumed fish oil (DHA only) supplements, but did find a decrease in IL-6 (20). Two more ESRD studies did not report any changes in CRP after twelve months (21) and three months of supplementing n-3 in ESRD patients, but doses were small in each study (22).

Many studies have been conducted in ESRD patients with n-3 fatty acids, but not many have looked at the pro-inflammatory cytokines. Yet, studies have

found favorable outcomes on pro- inflammatory markers in other populations. CKD patient populations from stage 2-5 would also greatly benefit from continued research with the use of n-3 fatty acids. Finally, our purpose is to evaluate the effects of fish oil supplementation on inflammatory markers, CRP, IL-1β, IL-6, TNF-α, in Chronic Kidney Disease patients.

Part 3: Methodology

Subjects

Fifty Chronic Kidney Disease (CKD) patients, both male and female, stage 3-5 and ≥ 18 years of age will be needed to participate in this study. Patients will be recruited through Central Texas Nephrology Associates clinics in Central Texas under the supervision of Ronald Wilson, MD (Nephrologist) and through Waco Family Health Center under supervision of Jackson Griggs, M.D. (Family Medicine). Subjects will not be considered for the study if they have taken an omega-3 supplement within the last 3 months, have allergies for fish or roasted rice flour (placebo), have a current involvement in another dietary study, an active illness requiring hospitalization, their life expectancy is less than 3 months, malabsorption syndromes, pregnant, or had any change in body weight (≥ 10 lbs.) in the past 6 months. Patients age 18 and older meeting eligibility criteria will be informed of the requirements of the study. All eligible participants will be asked to provide oral and informed written consent based on university-approved documents and approval that was granted by the Institutional Review Board for Human Subjects of Baylor University. Additionally, all experimental procedures involved in the study will be conformed to the ethical considerations of the Helsinki Code. The participants will be explained the purpose of the research, the protocol to be followed, and the experimental procedures to be used.

Study Site

All supervised data collection will be conducted at the Family Health Center located in Waco, TX, 76707. Blood samples will be transported from the Family Health

Center to the Exercise and Biochemistry Nutrition Lab (EBNL) at Baylor University in Waco, TX, to be analyzed.

Independent and Dependent Variables

The independent variable used throughout this study will include the nutritional supplementation of "Super Omega-3"(4 capsules contain 1400 mg of EPA, 1000mg DHA, 600 mg of Olive Fruit Extract and 20 mg sesame seed ligan extra, provided by Life Extension, Inc) and placebo supplements (Roasted Rice Powder, 670 mg, provided by Life Extension, Inc.). Dependent variables will include height, weight, quality of life (as determined by the SF-36 QOL questionnaire), inflammatory markers that will include interleukin (IL) – 6, tumor necrosis factor (TNF) - α, C - reactive protein (CRP), and IL-1β.

Entry/Familiarization Session

Participants expressing interest in participating in this study will be interviewed on the phone and/or in person by their primary care physician to determine whether they appear to qualify to participate in this study. Participants believed to meet eligibility criteria will then be invited to attend an entry/familiarization session. Once reporting to the clinic, participants will complete a medical history questionnaire and undergo a general physical examination by a physician to determine whether they meet eligibility criteria. Participants meeting entry criteria will be familiarized to the study protocol by way of a verbal and written explanation outlining the study design. At the conclusion of the entry/familiarization session, each participant will be given an appointment time to begin the study.

Experimental Protocol

This experimental protocol will be based on the protocol established by Schmitz et al. (Journal of the American Society of Nephrology, 2002). Table 1 presents the experimental design to be used in this study. Subjects will be familiarized as to the experimental procedures and sign informed consent statements in compliance with the Baylor University and *Central Texas Nephrology Internal* Review Board for the Protection of Human Subjects in Research and *Providence Hospital, Waco Texas, Institutional Review Board*. Prior to the study commencing, a full physical and cardiovascular examination will be performed by a physician. The participants' diets will not be standardized and subjects will be asked not to change their dietary habits during the course of the study. In a double-blind, placebo-controlled design, participants will be randomly chosen to receive either "Super Omega-3" or placebo supplement (roasted rice powder). Participants will be required to attend four testing sessions. At each testing session, participants will undergo an assessment that will determine weight, height, quality of life, and serum inflammatory markers. Patients will donate approximately 50 milliliters of blood, per physician's request, for analysis at each testing session. At the initial testing session, participants will be given a one-month supply of either "Super Omega-3" or placebo. Subjects will ingest two capsules twice day for 28 days with meals. Following 28 days of supplementation, participants will return to the clinic for a second testing session with their empty supplement bottles and receive an additional 28 days' worth of supplements. Following this phase of the study, participants will again return to the clinic for a third testing assessment, along with their supplement bottles and receive their final 28 days' worth of supplements to complete the study. Supplementation

compliance will be monitored by having the subjects return empty bottles of the supplement at the end of 4, 8, and 12 weeks of supplementation. Subjects will also be provided a supplement log to complete during the supplementation period to assist with documenting compliance to the protocol. The standard practice of pill counting will also be used to assess compliance. It has been recommended by some study authors that the standard for compliance should be between 80-100% (3, 4). Therefore, patients that consume 90% of the issued supplements in this study will be considered compliant.

Super Omega-3 (Fish Oil) and Placebo Composition

"Super Omega-3" and placebo are quality assured and quality controlled by Life Extension (Ft. Lauderdale, FL). Four capsules of "Super Omega-3" contain 1400 mg of EPA, 1000mg DHA, 600 mg of Olive Fruit Extract and 20 mg Sesame seed ligan extra. The placebo is composed of sesame oil.

Assessment of Body Mass and Height Measurements

Body mass and height will be measured at baseline and body mass will be measured again at four (4) weeks, eight (8) weeks and at twelve (12). Measurements will be taken by a doctoral student or a trained volunteer on a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale and a standard stadiometer for measuring height.

Blood Collection

Participants will donate approximately 50 milliliters of fasting venous blood, per physician's request, during each of the 4 testing sessions into five 10 ml serum separation vacutainer tubes (red tops) and one EDTA vacutainer tube (purple top). Blood samples will be obtained after a 12-hour fast and standardized to the same time of day for each sample. Blood samples will be obtained by inserting a needle into an antecubital vein

using standard phlebotomy procedures by study personnel trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. Study personnel will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Blood samples will be allowed to stand at room temperature for 10 min and then centrifuged. The blood collection tubes will be labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and will be wearing personal protective clothing) will centrifuge the serum samples at 2,400 rpm for 10 minutes, transfer serum into labeled serum storage containers, and store at -20°C for later analysis.

Serum Cytokines and Serum CRP

Serum will be analyzed in the Exercise Biochemical Nutrition Lab (EBNL) at Baylor University with separate enzyme-linked immunosorbent assay (ELISA) kits to determine levels of cytokines (IL6, IL1β, TNF-α). This will be conducted by Erika Deike, along with help from other members of the committee. Serum hs-CRP will be measured by a high sensitivity ELISA (Quest Diagnostics). hs-CRP will be determined by the Nephelometric method utilizing latex particles coated with CRP monoclonal antibodies. This ELISA assay was standardized against the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) /College of American Pathologists (CAP) CRP reference preparation.

Medical Outcomes Survey (MOS SF-36)

The MOS SF-36 is a questionnaire that covers 40 concepts related to health and was developed and used in the Medical Outcomes Survey. The SF-36 is a generic instrument which measures health-related quality of life (HRQOL) by

assessing eight different dimensions: physical functioning (10 items), role limitations caused by physical health problems (4 items), bodily pain (2 items), general health perceptions (6 items), energy/fatigue (4 items), social function (2 items), role limitation caused by emotional problems (3 items), and emotional well-being (5 items). The items are scored, with the higher score representing better HRQOL. Validity and reliability has been demonstrated in patients with chronic renal failure.

Reported Side Effects from Supplements

At the conclusion of each of the two testing sessions, participants will report by questionnaire whether they tolerated each supplement. In addition, participants will report any medical problems/ symptoms they may have encountered throughout the duration of the study through weekly phone calls. However, if symptoms/complications do arise prior to completing the questionnaire, participants will be encouraged to report them as they occur.

Statistical Analysis

The Statistical Package for the Social Sciences software for Windows (version 16.0, SPSS Inc, Chicago, IL) will be used to perform the statistical analysis of the data. The primary outcome variables are IL-6, IL-1β, TNF-α, and hsCRP. Repeated Measures ANOVA will be used to measure changes in the primary outcome variables using a 2 (fish oil or placebo) x 4 (time points) design. A Kolmogorov-Smirnov test for normality will be used to test all variables for normal distribution. A post hoc test will be used if needed to determine if

gender differences or differences among the stages of CKD existed across variables. Significance level is determined a priori at $p \leq 0.05$.

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Research Team

Erika Deike, M.Ed. Ms. Deike is a current Doctoral level research assistant in the ESNL and teaching assistant. She will serve as the principal investigator of the study assisting with all aspects of data collection and analysis.

Rodney Bowden, PhD. Dr. Bowden serves as the Associate Dean for Graduate Studies and Research in the School of Education and Associate Professor of Health Education in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Bowden will serve as co-chair of the dissertation.

Matt Cooke, Ph.D. Dr. Cooke serves as an Assistant Professor of Exercise Physiology and Nutrition. Dr. Cooke will serve as co-chair of the dissertation.

Darryn Willoughby, PhD, FISSN. Dr. Willoughby is an Associate Professor in the Department of HHPR at Baylor University and Director of the Exercise Biochemical Nutrition Lab.

Alexander Beaujean, Ph.D. Dr. Beaujean is an Assistant Professor in the Department of Educational Psychology. Dr. Beaujean will provide guidance for the statistical design and interpretation.

Ronald L. Wilson, MD. Dr. Wilson is a Nephrologist with the Brazos Kidney Center and Central Texas Nephrology Associates. Dr. Wilson will facilitate the research project at each of the six dialysis clinics in the Central Texas area. Dr. Wilson serves as medical supervisor for the ESNL and Center for Exercise, Nutrition & Preventive Health Research (CENPHR).

Rafer Lutz, Ph.D. Dr. Lutz is currently serving as Interim Chair and serves an Associate Professor in the Department of HHPR at Baylor University.

Jackson O. Griggs, M.D. Dr. Griggs is a family practitioner and faculty instructor at the Waco Family Medicine Residency Program. Dr. Griggs will

assist in the development of the methodology and procedures, and facilitate the research at the *Waco Family Health Center*.

Table 1. Overview of Research Design

T1 Week 0	T2 Week 8 (60 days)
Informed Consent	Fasting Blood Samples Obtained
Personal History/Medical History Form	Return Empty Pill Bottles
General Exam to Determine Qualifications to Participate in Study	Weight Measurements
Fasting Blood Samples Obtained	Quality of Life (SF36)
Height and Weight Measurements Quality of Life (SF36)	Side Effects Questionnaire
Side Effects Questionnaire	Meet with Physician (if needed)
Randomized, double-blind assignment to consume either "Super-Omega-3 or safflower oil	
Schedule 8-week Appointment	
Meet with Physician	

Procedures

Medical Monitoring. Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a mandatory medical exam by a physician to determine whether the subject meets entry criteria to participate in the study. This exam will include evaluating the medical history questionnaires. A telephone is in the clinic in case of any emergencies, and there will be no less than two researchers working with each subject during testing sessions. In the event of any unlikely

emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts 911. Instructions for emergencies are posted in the clinic in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during the course of the study to Rodney Bowden, PhD. If clinically significant side effects are reported, the participants will be referred to discuss the problem with Ronald Wilson, MD or Jackson Griggs, MD for medical follow-up. Dr. Wilson is a nephrologist and is an adjunct Professor in the Department of HHPR, while Dr. Griggs practice Family Medicine at the Family Health Center. Both have agreed to provide medical support and consultation for this study. Dr. Wilson and/or Dr. Griggs will evaluate the complaint and make a recommendation on whether any medical treatment is needed and/or whether the participant can continue in the study. If Dr. Wilson and or Dr. Griggs feel medical follow-up is necessary, the participant will be referred to obtain medical treatment from their personal physician. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Reported Side Effects from Supplement Questionnaires. At the beginning and end of the study, participants will report by questionnaire whether they tolerated the supplement, as well as report any medical problems/symptoms they may have encountered throughout the protocol of the study. However, if symptoms and/or complications do arise prior to completing the questionnaire, participants will be encouraged to report them as they occur.

Blood Samples. Participants will donate approximately 50 mL of fasting venous blood, per physician's request, during each testing session into five serum separation vacutainer tubes (red tops) and one EDTA vacutainer tube (purple top). Blood will be drawn by inserting a needle into the antecubital vein using standard phlebotomy procedures by study personnel trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. Study personnel will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. The blood collection tubes will be labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and will be wearing personal protective clothing) will centrifuge the serum samples, transfer serum into labeled serum storage containers, and store at -20°C for later analysis. Subjects will be seated in a phlebotomy chair. A tourniquet will be applied high on the brachium (upper arm) and will be tight enough to visibly indent the skin, but not cause the patient discomfort. The entry site will be thoroughly cleaned with an alcohol prep pad and allowed to dry. The participant will be instructed to lower their arm and make a fist several times in order to maximize venous engorgement. The appropriate vein will be selected. If a suitable vein is difficult to identify, the pads of the first and second fingers will be used to "slap" the veins gently to help dilate them. Alternately, the arm may be covered with a warm, moist compress to help with peripheral vasodilatation. If after a meticulous search no suitable veins are found, then the tourniquet will be released from above the elbow and placed around the forearm to search in the distal forearm, wrist and hand. If still no suitable veins are found, then the other arm will be checked taking extreme care to stay away from arteries, which are pulsatile. Once a suitable vein has

been found and blood has been drawn, the tourniquet will be released. Gentle pressure will be applied over the vein with a gauze pad, just proximal to the entry site to prevent blood flow. The needle will be removed and disposed in an appropriate sharps container.

Equipment

Digital Scale. Total body weight will be determined using a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills are not required to measure body weight.

Serum Cytokines and Serum CRP. Using enzyme-linked immunoabsorbent assays (ELISA), serum samples will be assayed for IL-6, TNF-α and IL1β. These samples will be assayed using a Wallac Victor-1420 microplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at a 450 nm wavlength against a known standard curve. Serum hs-CRP will be measured using a high sensitivity ELISA (Quest Diagnostics). hs-CRP will be determined by the Nephelometric method utilizing latex particles coated with CRP monoclonal antibodies. This ELISA assay was standardized against the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)/College of American Pathologists (CAP) CRP reference preparation.

Participants

Recruitment

Fifty CKD, stage 3-5, male and female patients > 18 years of age will be allowed to volunteer to participate in this proposed study. Participants will be recruited from Central Texas Nephrology Associates clinics in Central Texas under the supervision of Ronald Wilson, MD (Nephrologist) and Family Practice Clinic under supervision of

Jackson Griggs, M.D. (Family Physician), both in Waco, Texas. Dr. Wilson and Dr. Griggs will provide recruitment flyers to their patients and will ask for volunteers.

Selection Criteria

Participants will not be allowed to participate in this study if they:

1. Have allergies for fish or placebo
2. Are pregnant;
3. Have an active illness that requires hospitalization;
4. Have taken an omega-3 supplement within the last 3 months
5. Have a life expectancy $\leq$ 3 months;
6. Have a malabsorption syndrome;
7. Have had any change in body weight ($\geq$ 10 lbs.) over the previous 6 months.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., empty pill bottles) in the study will receive information regarding results of these tests if they desire. If subjects are Baylor Students, they will not receive any academic credit for participating in this study.

Potential Risks

The anti-inflammatory supplement to be investigated in this study has not yet been studied for the treatment of inflammation in CKD patients. It should be noted that recent research has demonstrated that oral administration of various anti-inflammatory compounds found in Super Omega-3 are not associated with any significant medical side effects. Moreover, this supplement is currently available in over the counter nutritional supplements sold in the United States. As with the vast majority of nutritional supplements, however, the FDA may not have evaluated the safety or marketing claims of Super Omega-3. Risks

associated with taking fish oil supplements may include an upset stomach, intestinal gas, and a fish oil taste in your mouth following administration.

Participants will donate approximately 50 milliliters of venous blood four (4) times during the study by way of a needle inserted into an antecubital vein using sterile techniques by study personnel trained in phlebotomy using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn.

Researchers involved in collecting data represent trained, non-physician, certified exercise specialists (Certified Strength & Conditioning Specialists, and/or American College of Sports Medicine Health Fitness Instructor$_{SM}$, Exercise Technologist$_{SM}$, or Exercise Specialists$_{SM}$), and physicians with appropriate medical specializations. All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) are located in the clinic in case of any emergencies and there will be no less than two researchers working with each subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts a doctor on staff at the clinic. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is that if the nutritional supplement is effective then this will provide some amount of evidence as to the effectiveness of Omega-3 in decreasing inflammation in CKD patients. In addition, participants may also gain insight about their health from the assessments performed. However, even if no individual benefit is obtained, participating in this study will help to determine whether ingesting this nutritional supplement affects anti-inflammatory markers. This information will be helpful to patients and doctors alike, to know whether the Omega-3 supplement is effective or not.

Assessment of Risk

The full potential medical benefits of Omega-3 supplementation are not yet known. Although, the various compounds contained in this supplement are available in a number of over the counter nutritional supplements. Therefore, the risk of supplementation of these compounds at the levels to be evaluated in this study is low. Therefore, the potential benefits of participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the

extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. However, participants will not be individually identified unless they give their written consent.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, International Society of Sports Nutrition, Experimental Biology, etc.) and published in a peer reviewed scientific journal (e.g., Nephrology (Carlton) Journal, Journal of Sport Science and Medicine, International Journal of Sport Nutrition and Exercise Metabolism, etc.).

Statement on Conflict of Interest

Funding for this study will occur through a donation of the omega-3 and placebo supplements from Life Extension, Inc. (Fort Lauderdale, FL) to Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Part 4: Informed Consent

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation

Informed Consent Form

Title of Investigation:	The Effects of Omega-3 Fatty Acids on Inflammatory Markers in Chronic Kidney Disease Patients
Principal Investigator:	Rodney G. Bowden, PhD Associate Dean, School of Education Baylor University
Co-Principal Investigators:	Erika Deike, M.Ed., Department of HHPR, Baylor University
	Ronald Wilson, MD, Medical Supervisor, *Central Texas Nephrology Associates*.
	Jackson Griggs, MD, Medical Supervisor, *Family Health Center*
Sponsors:	Life Extension, Inc.

Rationale:

Many chronic diseases are associated with low-grade systemic inflammation, including Chronic Kidney Disease (CKD) patients. They experience a multitude of conditions that are associated with the development of systemic inflammation, which can negatively affect their quality and length of life. But, study authors have reported that the use of omega-3 (n-3) fatty acids, found in fish oil, lowers systemic inflammation. Data suggests that a low level of n-3 fatty acids in the blood of CKD patients may contribute to the many inflammatory complications, including dialysis access thrombosis and cardiovascular outcomes. Additionally, the American Heart Association recommends that patients with high risk for cardiovascular morbidity and mortality, such as CKD patients, consume at least 1g of fish oil daily as well as maintain a dietary ratio of 4:1 for omega-6 to omega-3. In contrast, an abundance of omega-6 (n-6) fatty acids in the diet are known to increase inflammation, thrombosis and insulin resistance. It is believed that the benefits of n-3 fatty acids are found in its role in reaching a balance with n-6 fatty acids.

Initials_____

Study authors have concluded that the n-6/n-3 ratio should be about 1-4/1 rather than the ratio of 20-16/1, which is currently the makeup of the Western diet. A large consumption of dietary n-6 fatty acids, leads to oxidation of low-density lipoprotein (LDL), platelet aggregation, and thereby increasing inflammation.

Many studies have been conducted in CKD patients with n-3 fatty acids, but not many have looked at inflammatory markers. Yet, studies have found favorable outcomes on inflammatory markers in other populations. CKD patient populations would greatly benefit from continued research with the use of n-3 fatty acids. Therefore, our purpose is to evaluate the effects of omega-3 fatty acids (fish oil) supplementation on inflammatory markers in CKD patients.

Description of the Study:

I will be one of approximately 50 Chronic Kidney Disease patients over the age of 18 years who will participate in this study. Based on my doctor's diagnosis, I currently have Chronic Kidney Disease and/or are undergoing dialysis, and have higher than normal amounts of inflammation,. This study will be conducted by a doctoral student (Erika Deike) and other researchers (Dr. Rodney Bowden and Dr. Matt Cooke) at Baylor University, in addition to Dr. Ronald Wilson of *Central Texas Nephrology Associates*, and Dr. Jackson Griggs, *Family Health Center*.

During an initial familiarization session, I will be informed of the requirements of the study and sign an informed consent statement (this form) in compliance with the Human Subjects Guidelines of Baylor University. I will complete a medical history questionnaire and undergo a general physical examination to determine whether I meet eligibility criteria. If I am eligible to participate in the study, I will be familiarized to the study protocol by way of a verbal and written explanation outlining the study design. At this time I will be instructed that I will be required to report to Family Practice Clinic to participate in four separate testing sessions where my height and weight will be measured, fasting blood samples will be taken, and I will complete two questionnaires. After the first testing session, I will be randomly assigned to ingest an Omega-3 supplement or placebo (roasted rice flour) for 12 weeks. This familiarization session will take approximately 30 minutes to complete. Once I complete the familiarization session, I will be scheduled for the next testing session and at that time be instructed to refrain from exercise for 48 hours prior to baseline testing. At this time, I will be scheduled to return to the lab to go through testing procedures.

I understand that I will then donate 50 milliliters (approximately 5 tablespoons) of venous blood by inserting a needle into my arm using sterile techniques by an experienced technician using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the antecubital vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. This will be taken after I have fasted for twelve (12) hours.

Initials_____

I understand that I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that personnel who will be inserting the needle and taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. The process of inserting the needle and blood draws at each sampling point will take about 15 minutes. I understand that over the course of the study that I will have a total of four (4) blood draws (baseline, at four (4) weeks, at eight (8) weeks and at twelve (12) weeks), 50 milliliters each time.

I understand that I will also be asked to take either two (2) fish oil supplements, twice a day, (4 total) or two (2) placebo pills, twice a day, (4 total) during meals for twelve (12) weeks. The fish oil supplement is believed to help lower inflammation, helping to improve quality of life and possibly decrease risk of cardiovascular disease. Risks associated with these supplements may include an upset stomach, intestinal gas, and a fish oil taste in your mouth following administration.

I understand that I will complete a questionnaire (SF-36) four (4) times during the research project (beginning of the research project, at four weeks, at eight weeks and at twelve weeks). The questionnaire will be completed during my scheduled blood draw and should take approximately 20 minutes. These questionnaires will measure an increase or decrease in my quality of life during this research project. My height and weight will also be taken at each session to record any changes during the study.

I also understand that after each testing session is complete I will be required to complete a report of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study. Risks associated with these supplements may include a fishy after taste, an upset stomach, intestinal gas, and/or loose stools. I understand that if clinically significant side effects are reported, I will be referred to discuss the problem my doctor to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outlined by the investigators; 2) show up to all scheduled testing times; and 3) take the supplements as instructed. I agree not to take any other nutritional supplements during this study other than those prescribed by my doctor. In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any other nutritional supplements or medications during the course of the study that may affect vitamin/mineral status, blood inflammatory, or blood antioxidant levels that I may be removed from the study.

Initials_____

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: have taken an omega-3 supplement within the last 3 months, fish allergies or for placebo, a current involvement in another dietary study, an active illness requiring hospitalization, a life expectancy less than 3 months, malabsorption syndromes, pregnant, or had any change in body weight (≥ 10 lbs) over the past 6 months.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Erika Deike, M.Ed. (254-710-3241 or erika_deike@baylor.edu). I understand that if I experience any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with Dr. Ronald Wilson or Dr. Jackson Griggs to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that studies have evaluated the effects of using fish oil to control inflammation. I understand that using fish oil as a means to control inflammatory markers is a new approach that has been used in other studies. In addition, I understand that I may have side effects from this study that I will need to report to my doctor which may include nausea, intestinal gas and a fish oil taste in my mouth. Although the dose of fish oil is not excessive it may be higher than I am accustomed to using.

I also understand that I will have about 50 milliliters of blood, per physician's request, drawn from my antecubital vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist four (4) times during this study. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn.

I understand that this will occur during my scheduled appointments with a physician, a doctoral student or a trained volunteer. I understand that there will be researchers and a doctor working with me during each session for the length of the study. I understand that emergency procedures are posted in the clinic in the event that any emergency may arise.

I understand that the main benefit I may obtain from this study is that if fish oil is effective in controlling markers for systemic inflammation,

Initials _____

I may be able to use this supplement to decrease my risk for heart disease. I may also gain insight about my health from the assessments to be performed. However, even if no individual benefits are obtained, participating in this study will help to determine the impact that the use of fish oil may have on inflammation.

Alternative Treatment

This is not a medical treatment. Therefore, if additional medical treatment is needed, I must continue to obtain treatment for any medical problem I might have from my personal physician.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including questionnaires, medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study, which may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities.

I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood samples are analyzed that they will be discarded.

Initials: _____

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, my decision to withdraw from the study will not affect my future relations with Baylor University, Central Texas Nephrology Associates, or physician or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study.

I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that funding for this study was obtained by way of a product donation from Life Extension Inc. (Fort Lauderdale, FL) to Baylor University. I understand that researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Erika Deike, Department of Health, Human Performance, and Recreation, P.O. Box 97313, Baylor University, Waco,

Initials: _____

Texas 76798, phone number 254-710-3241 or e-mail at erika_deike@baylor.edu or by one of the research associates. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation.

I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research participant in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Matt Stanford, Ph.D., Associate Professor of Psychology and Neuroscience, P.O. Box 97334, Waco, TX 76798-7334, phone number 254-710-2961.

If you have additional questions during the study, please contact Erika Deike, Department of Health, Human Performance, and Recreation, P.O. Box 97313, Baylor University, Waco, Texas 76798, phone number 254-710-3241 or e-mail at erika_deike@baylor.edu.

You are making a decision whether or not to participate. Your signature indicates that you have read or been read the information provided above, understand and accept the possible risks, and have voluntarily decided to participate.

_____ _____

Signature of Subject Date

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

_____ _____

Signature of Investigator Date

Informed Consent Form Checklist

When using humans as subjects in research you must obtain their signed informed consent. Check each of the following items as they appear on your Informed Consent Form:

√ (a) A statement explaining the purpose of the research.

√ (b) A statement of the expected duration of the subject's participation.

√ (c) A description of the procedures to be followed.

√ (d) A description of any reasonable foreseeable risks or discomforts to the subject, including invasion of privacy.

√ (e) A description of any benefits resulting from the research, either to the subject or others.

√ (f) A statement that informs subject of his/her right not to be a subject in a research project that is also a teaching exercise.

√ (g) A statement informing subject about how his/her anonymity will be guarded; i.e., that their confidentiality will be protected by assigned code numbers, by limitations of who has access to data, by data storage in locked cabinets, by locked computer files, etc.

√ (h) A statement that the subject's participation is voluntary and that his/her refusal to participate will involve no penalty or loss of benefits to which the subject is otherwise entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.

N/A (i) A statement if applicable regarding the use of the Internet to collect data.

N/A (j) For research involving more than minimal risk, an explain action regarding the availability of any compensation or any medial treatment if injury occurs (if applicable, see OPRR Reports).

√ (k) If written informed consent is required, a place for the subject to sign and date the form, a place for the signature of a witness (preferably other than the principal investigator); and a statement that a copy of the signed consent form will be given to the subject for his/her records.

N/A___ (l) If the subject is a minor, a statement of parental responsibility in consenting to the child's participation in the study with a place for the parent to sign and date the form in addition to the participant's signature.

√_____ (m) The name, address, and telephone number of the principal investigator of the research project, and his affiliation with Baylor University. If the principal investigator is a graduate student, the name and telephone number of the faculty sponsor is also required.

√_____ (n) A statement informing the subject that inquiries regarding the nature of the research, the rights as a subject, or any other aspect of the research as it relates to his/her participation as a subject, can be directed to Baylor's University Committee for Protection of Human Subjects in research. The chairman is Dr. Matthew Stanford, Professor in Department of Psychology and Neuroscience Executive Associate Dean, School of Education, P.O. Box 97304, Waco, Texas 76798-7304, phone number 254-710-2961.

APPENDIX B

Medical History Questionnaire

BAYLOR UNIVERSITY ESNL

Medical History Inventory

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is CONFIDENTIAL as described in the Informed Consent Statement.

Name: _____ Age_____
Date of Birth_____

Name and Address of Your Physician:_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in the blank).

____Heart murmur, clicks, other cardiac findings?
___ Frequent extra, skipped, or rapid heartbeats?
____Chest Pain of Angina (with or without exertion)?
____High cholesterol?
____Diagnosed high blood pressure?
____Heart attack or any cardiac surgery?
____Leg cramps (during exercise)?
____Chronic swollen ankles?
____Varicose veins?
____Frequent dizziness/fainting?
____Muscle or joint problems?
____High blood sugar/diabetes?
____Thyroid Disease?
____Low testosterone/hypogonadism?
____Gluacoma?

____Asthma/breathing difficulty?
____Bronchitis/Chest Cold?
____Melanoma/Skin Lesions?
____Stroke or Blood Clots?
____Emphysema/lung disease?
____Epilepsy/seizures?
____ Rheumatic fever?
____ Scarlet fever?
____Ulcers?
____Pneumonia?
____Anemias?
____Liver or kidney disease?
____Autoimmune disease?
____Nerve disease?
____Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?
_____
_____

Please provide any additional comments/explanations of your current or past medical history.

_____
_____

Please list any recent surgery (i.e., type, dates etc.).

_____
_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

What was the date of your last complete medical exam?

Are you pregnant or trying to get pregnant? Please circle one (Yes, No)

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study? (Including strength and maximal exercise tests) ____

If yes, please explain:

Recommendation for Participation

____ No exclusion criteria presented. Participant is *cleared* to participate in the study.

____ Exclusion criteria is/are present. Participant is *not cleared* to participate in the study.

Signed: _____ Date: _____

APPENDIX C

Subject Recruitment Announcement

YOU ARE INVITED!

If you have kidney disease, you are at high risk for heart attack. This is an invitation to be a part of a study that will help doctors fight heart disease in patients with kidney disease. There are many reasons to join the study:

- FREE consultation with a nephrologist (a kidney specialist)
- FREE medication[1] if you are in the treatment group
- FREE studies that will help show your risk for a heart attack

The study will take place entirely at Family Health Center. It will not cost you any money to be involved. If you would like to accept this invitation, please call (254) 750 – 8290, and let us know. If you are not sure if you have kidney disease, ask your doctor: you qualify if your creatinine (a blood test) is 1.5 or more.

[1] Study medications are provided free of charge if you are in the treatment group. Depending on the study you are in, you may receive allopurinol, fish oil, or the anti-inflammatory medications curcumin and bosweillia.

APPENDIX D

Personal Information Sheet

Baylor University
Effects of Supplements (fish oil, CB, allopurinol) in CKD patients

Study: Fish Oil CB Allopurinol (Please circle one) Staff Initials: _____

Demographics:
Name: _____ Testing Session: _____ Testing #: _____
Date: _____ D.O.B.: _____ Age: _____

Testing Measures: Staff Initials: _____
Informed Consent _____
Medical History _____
Side Effect Questionnaire: _____

Height: _____in.
Weight: _____lbs.
Blood Pressure: _____mmHg
HR _____
Waist: _____in.

Pills remaining: _____ Compliance percentage: _____ (Not Applicable to T1)
Date of Next Appointment: _____
Date of Blood work: _____

Please check off once labs are complete:
Date: _____
Heparin tubes (4 green top) _____
SST (1 tiger top) _____

Notes:
_____
_____

APPENDIX E

Side Effect Questionnaire

Reported Side Effects From Supplement Questionnaire

Subject #: _____ Date: _____ Date: _____

Testing Session	1	2
Are you training on schedule?		
Are you adhering to the supplementation protocol?		
Rate the *frequency* of the following symptoms according to the scale: 0 = none 1 = minimal (1-2 per/wk) 2 = slight (3-4 per/wk) 3 = occasional (5-6 per/wk) 4 = frequent (7-8 per/wk) 5 = severe (9 or more per/wk)		
Dizziness?		
Headache?		
Fast or racing heart rate?		
Heart skipping or palpitations?		
Shortness of breath?		
Nervousness?		
Blurred Vision?		
Any other unusual or adverse effects?		

Rate the *severity* of the following symptoms according to the scale : 0 = none 1 = minimal 2 = slight 3 = moderate 4 = severe 5 = very severe		
Dizziness?		
Headache?		
Fast or racing heart rate?		
Heart skipping or palpitations?		
Shortness of breath?		
Nervousness?		
Blurred Vision?		
Any other unusual or adverse effects?		
Upset stomach or Nausea?		
Change in bowel habits?		

PLEASE REMEMBER TO REPORT ANY SIDE EFFECTS IMMEDIATELY.

Directions: If necessary, please contact either Erika Deike, MEd. at 254-710-3241 or Rodney Bowden, Ph.D. at 254-710-4499 . You may also e-mail either at Erika_Deike@baylor.edu or Rodney_Bowden@baylor.edu. Thanks for your participation!

APPENDIX F

LifeExtension®
QUALITY SUPPLEMENTS & VITAMINS, INC.

CERTIFICATE OF ANALYSIS

Product Name:	Super Omega-3 EPA/DHA
Item Number:	00982
Description:	Oblong shaped, clear brown soft gelatin capsule containing brown colored liquid
Lot Number:	0111-8021
Manufacture Date:	January 2009
Best By Date:	January 2011

ASSAYS

TEST	SPECIFICATION (per softgel)	RESULT (per softgel)
Wild Fish Oil Concentrate	1,000 mg	103.0%
EPA (EE)	350 mg	118.6%
DHA (EE)	250 mg	104.0%
Polyphenol Oil (Extra Virgin Olive Oil)	120 mg	103.0%
Olive Fruit Ext. (Standardized)	15 mg	105.0%
Olea Select Olive Ext. (*Olea europa*, Fresh Fruit)	15 mg	105.0%
Sesame Seed Lignan 70% Ext.	5 mg	105.0%
Stabil Enhance (Rosemary Ext.)	1 mg	100.0%
Rupture Time Limit	<15 minutes	Complies
Average Weight	Variation ± 10%	1386 mg

MICROBIOLOGICAL TESTS

TEST	SPECIFICATION	RESULT
Total Plate Count	NMT 3,000 cfu/g	Complies
Yeast & Mold	NMT 300 cfu/g	Complies
Salmonella	Negative per 10 gram	Negative per 10 gram
Escherichia coli	Negative per 10 gram	Negative per 10 gram
Staphylococcus aureus	Negative per 10 gram	Negative per 10 gram
Pseudomonas aeruginosa	Negative per 10 gram	Negative per 10 gram

Reviewed and Approved by Life Extension Quality Assurance/Quality Control

Director of Quality Assurance/Quality Control

GLOSSARY

Apoptosis − the process of programmed cell death that may occur in multi-cellular organisms.

Creatinine − a product of normal muscle breakdown and urea is the waste product from the breakdown of protein.

Cytokines − such as interleukins and lymphocytes, are regulatory proteins that are released by cells of the immune system and operate as intercellular mediators in the assembly of an immune response.

Endothelium − A layer of flat cells lining the closed internal spaces of the body such as the inside of blood vessels and lymphatic vessels (that convey the lymph, a milky fluid) and the heart.

Glomerular filtration rate (GFR) − rate at which the kidneys filter blood.

Hyperkalemia − an elevated blood level of the electrolyte potassium.

Hypertriglyceridemia − denotes high blood levels of triglycerides, the most abundant fatty molecule in most organisms.

Interleukins − are a group of cytokines (secreted proteins/signaling molecules) that were first seen to be expressed by white blood cells (leukocytes).

Low-Grade Systemic Inflammation − inflammation that is not confined to a particular tissue but involves the endothelium and other organ systems.

Lymphokines − are a subset of cytokines that are produced by a type of immune cell known as a lymphocyte. They are typically produced by T cells to direct the immune system response by signaling between its cells.

Macrophages − a type of white blood cell within tissues, produced by the differentiation of monocytes, that ingests foreign material.

Monocytes − a type of white blood cell, part of the human body's immune system. They replenish resident macrophages and dendritic cells under normal states, and can move quickly (approx. 8-12 hours) to sites of infection in the tissues and divide/differentiate into macrophages and dendritic cells to elicit an immune response.

Nuclear factor-κβ (NF-κβ) − a protein complex that controls the transcription of DNA.

Pericarditis − inflammation of the pericardium (the fibrous sac surrounding the heart).

Resolvins − compounds that are made by the human body from the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Experimental evidence indicates that resolvins reduce cellular inflammation by inhibiting the production and transportation of inflammatory cells and chemicals to the sites of inflammation. They are released and used immunologically by the kidneys as a tool against acute renal failure, when it occurs.

Thrombosis − is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system.

Uremia − a term used to loosely describe the illness accompanying kidney failure (also called renal failure), in particular the nitrogenous waste products associated with the failure of this organ.

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