Fish Oil Supplements and Symptoms of the Common Cold in Healthy Young Women

by

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ABSTRACT

Background: Research in animal models suggests that fish oil ingestion may impair immunity and increase risk for infection. To date there are no studies examining this relationship between fish oil ingestion and risk for infection in humans.

Objective: The primary aim of this randomized, placebo-controlled, double-blind, parallel-arm study was to examine the effect of 400 mg of EPA and 200 mg of DHA, the main components of fish oil (FO) supplements, on the incidence of symptoms related to upper respiratory tract infections in healthy young females, at a large southwestern university.

Design: Healthy young women between 18 and 38 years of age who were non-obese (mean BMI 23.7 ± 0.6 kg/m^2) were recruited from an urban southwestern university campus. Subjects were non-vegetarians, non-smokers, and reported consuming less than one serving (3.5 oz) of fish per week. Participants (n=26) were randomized according to age, body weight, BMI, and daily n-3 fatty acid (FA) intake into two groups: FO (one gel capsule of 600 mg EPA/DHA per day) or CO (one placebo gel capsule of 1000 mg coconut oil per day). Participants completed a validated daily cold symptom survey, the Wisconsin Upper Respiratory Symptom Survey-21 for 8 weeks. Fasting blood samples measuring TNF-α concentrations were taken at weeks 1 and 8, when 24-hour dietary

recalls were also performed. Anthropometric measurements were recorded via bioelectrical impedance at trial weeks 1, 4, and 8.

Results: The 8-week trial of FO supplementation did not significantly change the average score for perception of cold symptoms between FO and CO groups (167 ± 71 and 185 ± 56, p=0.418, respectively). Plasma TNF-α levels (pg/mL) did not differ between groups (p=0.482). TNF-α levels were significantly correlated with body weight (r=0.480, p=0.037), BMI (r=0.481, p=0.037, and percent body fat (r=0.511, p=0.025) at baseline.

Conclusions: Healthy young women taking a fish oil supplement of 400 mg EPA and 200 mg DHA per day over 8 weeks does not impose unintentional health consequences. These findings do not refute the American Heart Association's current recommendations for all Americans to consume two servings (3.5 oz) of a variety of oily fish per week. Depending on the type of fish, this current recommendation equates to approximately 200-300 mg per day of EPA and DHA n-3 polyunsaturated fatty acids. Additional research is needed to investigate the effects of higher dosages of fish oils on daily cold symptoms.

DEDICATION

I dedicate this to all of my current and prior inspirational teachers, classmates, and mentors. I am grateful for the guidance and motivation you have given me to dream big and achieve my goals. This is for all of those who have paved the way before me and those who will be coming up next, I am privileged to be here and put forth my best effort.

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INTRODUCTION

In a 2007 national survey, 17.7 percent of American adults reported using a natural dietary supplement for health reasons, one that is not classified as either a vitamin or mineral (1). Natural remedies for the common cold such as Echinacea are likely to reduce the severity of respiratory tract infections however scattered reports show that some dietary supplements actually promote these infections (2,3). Supplements derived from fish oil, containing the essential fatty acids eicosapentaneoic acid (EPA) and docosahexaneoic acid (DHA), are the most commonly used natural supplement, used by 37.4 percent of Americans (1).

The anti-inflammatory effects of EPA and DHA in fish oil may have a beneficial role in reducing inflammation in chronic inflammatory diseases such as Crohn's disease, rheumatoid arthritis (4), and inflammatory bowel disease (5,6). There is also evidence, based on epidemiological and controlled-clinical trial research, that supports reduced cardiovascular disease incidences with omega-3 fatty acids (7). As part of a heart-healthy diet the American Heart Association recommends eating two 3.5 ounce servings of fish per week (8). Fish oil recommendations are also being promoted in popular media outlets such as the Dr. Oz show, which lists omega-3 fish oils as one of the top five supplements all women should take (9). Although there is support for the beneficial effects of fish oils in some chronic diseases, the fish oils EPA and DHA have

immunosuppressive effects that have been shown to increase susceptibility to infections in animals (10-12).

Schwerbrock et al. (12) noted that fish oil-fed mice had an increased mortality, increased viral load, and prolonged recovery period after influenza infection. Byleveld et al. (10,11) observed that mice fed fish oil diets had delayed viral clearance and decreased cytotoxic T cell activity. While these studies have indicated that fish oil supplementation is hazardous to the health of mice with influenza virus, a suppressed immune response has also been observed in mice consuming omega-3 polyunsaturated fatty acids (PUFAs) inoculated with *Listeria monocytogenes* and *Mycobacterium tuberculosis* (13,14). Although the prior literature suggests an ill-adaptive response for influenza viral clearance in fish-oil fed mice (10-12,14), there is a deficiency in the literature that looks at the immune response to cold and flu viruses in healthy humans consuming fish oil supplements. With the heightened awareness of omega-3 PUFAs to stop inflammation, it is not yet clear whether these dietary supplements can increase susceptibility to infections in healthy human subjects.

Research has shown a significant decrease of lymphocyte proliferation in healthy individuals consuming fish oil supplements containing as little as 1 g of EPA per day (15). Additional support of dietary omega-3 PUFAs delaying the immune response has been observed in slowed wound healing of blisters in individuals taking 1.6 g of EPA and 1.1

g of DHA for 4 weeks (16). These randomized, placebo-controlled, double-blinded studies suggest fish oil supplements may have detrimental effects on the immune system which raises concern since alternative medicine therapies are on the rise in the United States (1).

Purpose of the Study

The primary aim of this randomized, placebo-controlled, double-blind, parallel-arm study was to examine the effect of 400 mg of EPA and 200 mg of DHA, the main components of fish oil supplements, on the incidence of symptoms related to upper respiratory tract infections in healthy females, aged 18-40, at a large southwestern university.

Hypothesis

We hypothesized that self-reported levels of cold symptoms will increase in the fish oil supplement group compared to a placebo-control group. This study coincided with the highest occurrences of cold and flu symptoms which appear in January and February in the United States (17).

Definition of Terms

- BMI: [weight (in pounds) / height (in inches) x height (in inches)] x 703; < 18.5 is underweight, 18.5-24.9 is normal, 25.0-29.9 is overweight, and > 30 is obese (18).
- Common cold: an acute respiratory illness caused by viral infection of the upper respiratory tract. Symptoms typically appear one to three days after exposure to a cold virus. Symptoms are mild and

include runny or stuffy nose, sore throat, cough, sneezing, and congestion (19,20).

- Fish oil: derived from marine sources which are rich in the long-chain omega-3 PUFAs EPA (20:5n-3) and DHA (22:6n-3) (7).
- Healthy: no unresolved health conditions. Some prescription medications were excluded such as thyroid medications for an accompanying study and also any anti-inflammatory medications, e.g., corticosteroids.
- Regular smoker: greater than or equal to 10 cigarettes per day (21).
- Training athlete: engaged in purposeful exercise five or more times per week.
- Vegetarian: excludes all meat, fish, and poultry products from diet.

Limitations

- Subject compliance to protocol includes administration of 1 supplement and completing the cold symptom survey daily.
- Recording of dietary sources of omega-3 fatty acids occurred via a validated food frequency questionnaire (FFQ) specific to sources rich in omega-3 fatty acids.
- External factors such as lack of sleep or psychological stress may impact inflammatory state and susceptibility to infection.

- Short trial duration (8 weeks) may not accurately reflect the duration of the 2011-2012 cold and flu season and may not show any long-term physiological effects of omega-3 fatty acids.
- A small subject pool (n=26) limit the generalizability of the results and obtaining statistical significance.
- Study does not observe detailed mechanisms to determine specific mediators in the immune response.
- Perception of cold symptoms is a subjective measure. People may have different tolerances impacting their self-reported responses on the questionnaire.
- Cannot control exposure to cold and flu viruses.

Delimitations

Participants were limited to healthy college women, aged 18-38 years from a large southwestern university in the United States with BMI between 18.5 and 30. Exclusion criteria for the study included participants who are regular smokers, women who are pregnant or lactating, those who consume greater than or equal to 1 serving of fish per week, regular use of fish oil supplements, regular use of thyroid medication, corticosteroids and/or non-steroidal anti-inflammatory drugs, vegetarians, training athletes and/or individuals who are trying to lose or gain weight, those who have allergies to seafood, and individuals who have received a flu shot for the 2012 season.

Chapter 2

LITERATURE REVIEW

Fish Oils Overview

Western Diets & Fatty Acid Ratio. Dietary patterns in developed nations are associated with a high consumption of pro-inflammatory omega-6 (n-6) PUFAs and a reduced consumption of anti-inflammatory omega-3 (n-3) PUFAs (22). The ratio of n-6 to n-3 PUFAs in the Western diet is now 15 times greater than the diet humans have evolved upon in the past (23). The dietary intake of n-6 to n-3 PUFAs was much more balanced in hunter-gatherer societies with an estimated n-6:n-3 ratio of 1:1 (24). The ratio of n-6 to n-3 PUFAs has increased to 16-20:1 in the current Western diet and is a likely contributor to an increased incidence of CVD and inflammatory disorders (22,25,26).

The n-3 fatty acids, α-linolenic acid (ALA), EPA, and DHA compete as substrates for the prostaglandin pathway with the n-6 fatty acids, e.g. arachidonic acid (AA). Anti-inflammatory mediators increase with n-3 fatty acid consumption concomitant with decreases in the inflammatory mediators (5,27). The impact of the dietary ratio of n-3 and n-6 PUFAs is dose-dependent, modifies cellular composition within a few days, and reaches a new steady-state in up to four weeks (27). High intakes of n-6 PUFAs increase AA in the plasma and cell membranes and lead to the increased production of pro-inflammatory metabolites (22). The high n-6 to n-3 ratio is potentially linked to the pathogenesis of chronic diseases such

as cardiovascular diseases (CVD), inflammatory, and autoimmune disorders (22,23,25,28). Inflammatory and autoimmune disorders are characterized by elevated levels of the pro-inflammatory cytokine, interleukin 1 (IL-1) which is promoted by n-6 fatty acids (26).

Surveys from cross-sectional and epidemiologic research found an inverse correlation with n-3 fatty acid status and prevalence rates of CVD and depression. In 1996 the annual seafood consumption, assessed by pounds per person, was about three-fold higher in Japan (148 lb/person) compared to the United States (48 lb/person) (29). Statistics from the World Health Organization in 2008 showed the prevalence of CVD mortality among men aged 45-59 differed among regions such as Eastern (e.g., Japan and Singapore) and Western regions (e.g., United States and Canada). The Eastern region (23/100,000) had a 60% lower risk of CVD mortality compared to the Western region (37/100,000) (30).

Geographic regions have differing risk factors for CVD and n-3 fatty acid status. Counterintuitive to the previous report on CVD mortality risk among regions, Asian Indians in Singapore have a greater risk of CVD than any other ethnic group. The National University of Singapore Heart Study in 2004 was a cross-sectional survey which looked at the differences of essential fatty acid plasma concentrations among Asian Indians and Chinese men in relation to CVD susceptibility. In 292 subjects, Asian Indians showed lower total n-3 plasma fatty acid concentrations compared to the Chinese (4.71% versus 5.27%, $p<0.001$) which

correlated positively with HDL-C and Apo-A1 concentrations and negatively with LDL-C and Apo-B concentrations. These results support the premise that high n-3 fatty acid status is associated with cardio-protective benefits (31).

Other populations world-wide have greater n-3 fatty acid status compared to Western cultures. The native Greenland Eskimos' diets are characteristically high in n-3 PUFAs from consuming whales, seals, Greenland halibut, and salmon (32). The Eskimo population along with the Japanese, have diets high in marine n-3 fatty acids and low incidences of myocardial infarction and chronic inflammatory diseases compared to Western cultures (26,33). These correlations have been observed since 1971, when Bang et al. studied Greenland Eskimos who consume high amounts of animal fats but have low incidences of CVD compared to Danish controls consuming a typical Western diet. It was observed that Eskimos had much lower plasma triglycerides (TAG) ($p<0.001$) and pre-β-lipoprotein levels than controls ($p<0.001$) (32). Pre-β-lipoproteins are synonymous with very low density lipoproteins (VLDL) produced in the liver to transport endogenous TAGs to non-hepatic tissues (34).

Recently however, the Arctic Inuit populations' diet is changing in response to Western influences. In 2005 the Inuit Health in Transition study took a baseline survey of two Inuit populations, the Nunavik in northern Quebec and the Greenland Inuit, measuring the fatty acid profile of red blood cell phospholipids which is a good indicator of dietary fatty

acid intake. The mean total n-3 levels for the Greenland population was 12.1% compared to 9.4% for the Nunavik ($p<0.001$). There are many occupational, environmental, and generational factors that contribute to the complex dietary changes seen in this population, which is reflected in their *trans* fatty acid status. Average *trans* FA levels were three-fold higher in the Nunavik (1.20%) than the Greenland (0.43%) which may explain the displacement of n-3 FA consumption in their diets due to high *trans* FA intake (35).

Inverting the essential fatty acid ratio to favor dietary n-3 fatty acids exert immunosuppressive and anti-inflammatory effects in the body (23). N-3 fatty acids have anti-atherogenic properties such as decreasing platelet aggregation and lowering TAG levels (33). The deficiency of n-3 PUFAs in Western diets is likely a major contributor to the development of chronic diseases although this has not always been the case when examining the evolutionary aspect of foods containing EPA and DHA (23).

Over the last 100 years, modern agriculture and technological advancements in the food industry have contributed to the increased consumption of n-6 fatty acids (23,28). Anthropological and epidemiologic studies have examined the diet humans have evolved upon which indicated a n-6 to n-3 ratio of approximate 1 compared to current Western diets estimated near 15.0-16.7 to 1. The progress of increasing essential fatty acid ratio coincides with major changes in the type and amount of fatty acids consumed and antioxidant content in foods. In the past, the

food supply contained omega-3 PUFAs in eggs, meat, fish, wild plants, nuts, and berries (36). Theories contributing to the lower n-6 to n-3 ratio in hunter-gatherer societies were based on estimated levels of *trans*-fatty acid intake at a fraction of the current 2% of total calories. Also, PUFA intake was estimated at nearly twice the current amount (15 g/d) (37).

Marine-derived fish oils have high concentrations of the n-3 PUFAs, EPA and DHA (24,38). Sources of the plant-derived n-3 PUFA, ALA, include nuts, seeds, soybean and canola oils (24). Based on the evidence discussed above, the dietary balance of essential fatty acids is especially important for maintaining good health and development. The increased concern in the balance of essential fatty acids and the deficiency of n-3 fatty acids in Western diets has led to more recommendations to supplement the diet with n-3 fatty acids (25).

Biochemistry, Absorption, & Metabolism. The long-chain n-3 and n-6 PUFAs are important for their distinctive health functions which are due in part to their structure and properties. Unlike plants, humans cannot metabolically convert n-6 to n-3 fatty acids because mammals lack the omega-3 desaturase enzyme to produce ALA from AA, making them two distinct families (23). AA is part of the n-6 fatty acid family along with the other n-6 PUFA derivatives ɣ-linolenic acid (GLA), dihomo-ɣ-linolenic acid (DGLA), adrenic acid, and docosapentaenoic acid (DPA) which come from the precursor linoleic acid (LA). In the n-3 fatty acid family ALA is the precursor to the long-chain PUFAs EPA, DPA, and DHA (34,39).

Both long-chain PUFAs n-6 and n-3 are digested and efficiently absorbed by the small intestine (39). In particular, it was reported that ALA has an absorption rate greater than or equal to 96%. Several metabolic destinations for n-3 PUFAs include storage in adipose tissue, producing energy through β-oxidation, converting into other non-essential fatty acids, or enzymatically elongation and desaturation into long-chain PUFAs in the liver (22). N-6 PUFAs are metabolized similarly, where they are incorporated into phospholipid membranes, used in eicosanoid synthesis, or used for energy production via β-oxidation (39).

LA and ALA are considered essential in the diet because mammalian cells lack delta-12-desaturase and delta-15-desaturase which insert double bonds in oleic acid to produce LA and ALA (5,34). The presence of the elongase and desaturase enzymes for the delta-9 carbon and above allow for conversion of ALA and LA into EPA/DHA and AA respectively (40).

The first step in the metabolism of ALA to EPA and DHA is the addition of a fourth double bond to produce the intermediate stearidonic acid by delta-6-desaturase, which is considered the rate-limiting step. Following elongation and additional desaturation from delta-5-desaturase, EPA becomes the first product in ALA metabolism. Several other steps of elongation and desaturation occur to yield DHA, although only small amounts of DHA are produced despite available levels of ALA in body tissues. The conversion rate from ALA to EPA in humans is estimated at

8-20% whereas conversion from ALA to DHA is even smaller at 0.5-9% (22). Ultimately, the elongated n-3 fatty acids get incorporated into the cellular phospholipid membranes of all body tissues (22). In the phospholipid membrane, EPA produces biologically active eicosanoids that have immunosuppressive actions. These anti-inflammatory eicosanoids consist of series 5 leukotrienes and series 3 thromboxanes and prostaglandins, which exhibit beneficial effects in preventing arrhythmias and thrombosis (22,23,39).

LA metabolism into AA competes for the same elongase and desaturase enzymes as ALA. In fact, in vitro studies provide evidence that delta-6-desaturase has a preference for n-3 fatty acids over n-6 fatty acids (39). Dietary LA undergoes a desaturation reaction generating a third double bond by delta-6-desaturase to yield GLA. Elongation adds 2 carbons to GLA to produce DGLA. Delta-5-desaturase undergoes a desaturation reaction to produce AA (41). LA metabolizes into AA which is an important metabolite, structurally compromising approximately 15% of total fatty acids in erythrocyte membranes and is also involved in apoptosis (41,42). AA also has a major role as the precursor of many eicosanoids such as thromboxanes, prostaglandins, and leukotrienes within the cell (43).

Once incorporated into the phospholipid membranes of the cell, AA exhibits strong inflammatory responses mediated by biologically active eicosanoids. These metabolic products are involved in platelet

aggregation and thrombus formation which contribute to the progression of atherogenesis (23,39). To produce eicosanoids, AA first must be released from the *sn*-2 position in phospholipids by phospholipase A2 in response to a variety of stimuli. The cyclooxygenase pathway synthesizes prostaglandins and thromboxanes whereas 5-lipoxygenase synthesize leukotrienes (43).

Eicosanoids have varying physiologic functions and antagonistic effects. Prostacyclin (PGI2) increase cyclic adenosine monophosphate (cAMP) activity, thus acts as a platelet anti-aggregating factor. Conversely, thromboxane-A2 (TXA2) decreases cAMP activity and promotes platelet aggregation. Another opposing pair is prostaglandin E2 (PGE2) which acts as a blood vessel vasodilator and prostaglandin F2 (PGF2) which acts as a vasoconstrictor (34). These prostaglandins are produced in response to their respective fatty acid of the n-6 or n-3 families. AA competes with EPA in these metabolic pathways, displaces EPA's position in phospholipids, and invokes an inflammatory response; conversely, EPA metabolic products have a much weaker inflammatory response (25).

Sources & Recommended Intakes. Although ALA is the parent compound to all other n-3 fatty acids, EPA and DHA can also be provided from ingested fish oils. To produce EPA and DHA, ALA undergoes a series of desaturation and elongation steps in the liver. Oily fish such as salmon, mackerel, and tuna are rich sources of omega-3 fatty acids EPA and DHA (5,25). Different varieties of marine animals have varying amounts of EPA

and DHA per serving. For example, sockeye salmon has 600 mg of DHA and 450 mg of EPA whereas Atlantic salmon has 950 mg of DHA and 250 mg of EPA per serving (44). Aside from n-3 fatty acids in animal sources there are several plant sources high in the n-3 fatty acid ALA such as walnuts, flaxseed, rapeseed, and green leafy vegetables (22).

As seen in the n-3 family, the parent compound to all other n-6 fatty is dietary LA although it too can be provided from the diet in limited amounts (39). LA undergoes a similar pathway to produce AA in the liver. Dietary sources rich in n-6 fatty acid LA are corn, sunflower, and soybean oils (5) and preformed AA is found in small amounts in eggs, animal, and organ meats (22,45).

Based on the Dietary Reference Intakes (DRIs) established by the National Academy of Sciences, Acceptable Intakes (AIs) and Acceptable Macronutrient Distribution Ranges (AMDRs) have been set for essential fatty acids. The appropriate n-6 to n-3 ratio is 1-2:1 which is consistent with the recommended AIs (23). The AI for adult men 19-50 years, is 17 g/day of LA and 1.6 g/day of ALA. For adult men 51 years and older the AI is 14 g/day of LA and also 1.6 g/day of ALA. The AIs for adult women 19-50 years is 12 g/d of LA and 1.1 g/day of ALA. For adult women ages 51 years and older, ALA remains the same at 1.1 g/day whereas LA is 11 g/day. However, for pregnant and lactating women the AI is 13 g/day of LA and 1.4 g/day of ALA but during lactation it is slightly lower at 1.3 g/day of ALA (39).

AMDRs for n-6 and n-3 fatty acids are established based on epidemiological and intervention trials to provide sufficient amounts for preventing chronic diseases and nutritional deficiencies. To meet the AI for LA, the lower and upper boundary level is estimated at 5-10% of total energy. Likewise, the AMDR for ALA is an estimated 0.6-1.2% of energy. Because EPA and DHA have greater physiologic effects than ALA, a total n-3 AMDR cannot be determined therefore, EPA and/or DHA should be consumed at 10% of the AMDR for ALA (39).

Biological Functions. Long-chain PUFAs have important biological functions for growth, reproduction, and neurological development (40). N-3 fatty acids, EPA, DHA, and ALA are important for brain function and brain development especially for newborns (22). There are also vital roles of n-3 fatty acids for maintaining optimal cardiovascular health and function related to their anti-thrombotic, anti-atherogenic, vasoprotective, and anti-arrhythmic properties (22,46). N-6 fatty acids have been shown to have roles in normal epithelial cell function. AA functions as a substrate for eicosanoid production and is involved in cell signaling pathways. Aside from their physiological function, the long-chain PUFAs play a vital role as structural components of phospholipid membranes. They are also involved in cell signaling, expressing specific genes that control fatty acid synthesis and carbohydrate metabolism. In addition, the fatty acids may also be used as ligands for certain receptors that control inflammation, neurological function, and the development of adipocytes (39).

Role in Immune Function. PUFAs are integral to immune function. N-3 and n-6 PUFAs are incorporated into the phospholipid membranes of immune cells and have different effects on the fluidity and function of the cells. Leukocytes have varying functional changes through different mechanisms, which are influenced from the composition of dietary fatty acids (47). The most studied of such mechanisms are eicosanoid and cytokine synthesis, gene expression, lipid rafts, and resolvin synthesis (6,40,47).

As n-3 PUFAs are incorporated into immune cell membranes, they directly influence the immune response by replacing AA as the precursor for eicosanoid synthesis. Likewise, EPA and AA are competitors for the same enzymatic pathways (cyclooxygenase and 5-lipoxygenase) to produce inflammatory mediators. Compared to AA, eicosanoids produced from EPA have a significantly less inflammatory response and include prostaglandin E3 (PGE3), leukotriene B5 (LTB5), and series 3 thromboxanes (47).

The pro-inflammatory eicosanoids from AA include prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and series 2 thromboxanes (47). The n-3 PUFAs exhibit anti-inflammatory effects through inhibiting the production of small proteins called cytokines, which are also immune mediators. Pro-inflammatory cytokines are inhibited from n-3 PUFAs at the gene-transcriptional level, thus reducing the synthesis of the cytokines TNF-α, IL-1β, and IL-6 (47).

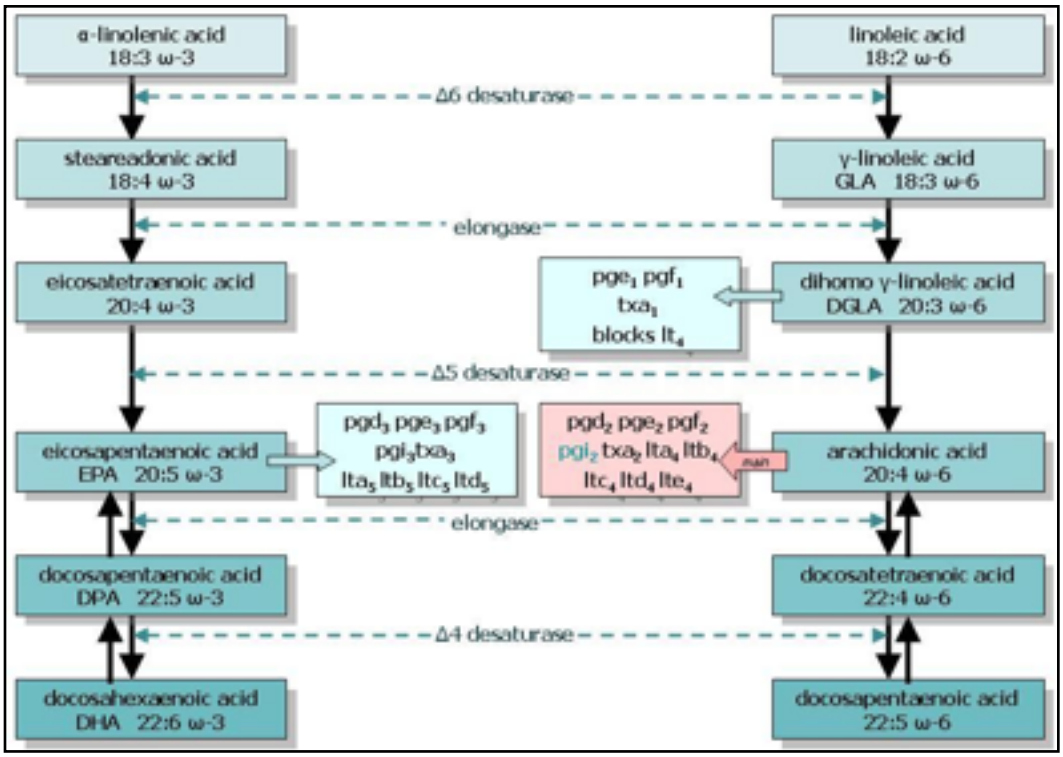

Figure 1. Metabolism of Essential Fatty Acids to Eicosanoids
Source: http://upload.wikimedia.org/wikipedia/en/0/09/EFA_to_Eicosanoid.JPG

EPA and DHA can mediate gene expression of immune cells through the activation of nuclear receptors such as the peroxisome proliferator-activated receptors (PPARs) (6,40). PPARs are competitors in the signaling pathways of the nuclear transcription factor kβ (NF-kβ), a regulator of genes involved in inflammation (25,40). The transcribed genes involved in the inflammatory response are cytokines, adhesion molecules, and other inflammatory mediators (47). PPARs are also responsible for the severity and length of the inflammatory response by changing gene expression of proteins that catabolize pro-inflammatory eicosanoids

(6,47). The indirect effects of n-3 PUFAs on the inflammatory response is through inhibiting pro-inflammatory pathways by activating the transcription of NF-kβ through toll-like receptors (47).

The alteration of cell membranes by n-3 PUFAs changes the fluidity, structure, and function of the receptors in the membranes as well as the eicosanoids that are secreted (6). Immune cell activation and proliferation uses the lipid rafts to aggregate several receptors. Lipid rafts are characteristically rigid in structure because of the included cholesterol and saturated fatty acid-rich phospholipids, however the presence of n-3 fatty acids alters the function through displacement of signaling proteins (47).

Resolvins and neuroprotectins are pro-resolving mediators derived from EPA and DHA that are biologically active in reversing inflammation (6,48). These mediators were originally discovered showing effects in the resolution phase of acute inflammation. There are two bioactive families of mediators, Resolvin E series (RvE) and Resolvin D series (RvD), which are derived from EPA and DHA, respectively (47,48). RvE1 is associated with decreasing pro-inflammatory cytokines IL-6 and TNF-α during the resolution phase and also inhibits NF-kβ activity. Neuroprotectin D1 (NPD1) from the RvD series, synthesized in the brain, is a strong anti-inflammatory mediator by inhibiting transcription of IL-1β genes in human glial cells (47).

The Immune Response

Physiology. The immune system is composed of a variety of cell types, tissues, and organs. Lymphoid tissues involved in the immune response include the thymus, spleen, and lymph nodes (5).

Immunity. There are two types of defense systems involved in the immune response. The innate immune system is present at all times in humans and helps defend against pathogens prior to entry and after penetration into tissues and fluids. The second class of the immune system is adaptive immunity which is alerted in response to a foreign antigen that has surpassed the innate immunity. Adaptive immunity can be divided into humoral and cellular immune responses which are mediated by different lymphocytes (40). Unlike innate immunity, adaptive immunity targets specific pathogens and operates quickly at subsequent exposures to a memory component.

White Blood Cells. Leukocytes are white blood cells involved in the immune system that can be further classified into phagocytes and lymphocytes (5). Phagocytes are innate immune cells and include the granulocytes, monocytes, and macrophages that function to clear pathogens as well as tissue debris (48). Lymphocytes are adaptive immune cells and are classified as T lymphocytes, B lymphocytes, and natural killer cells based on their specific role in immunity (5). B lymphocytes are mediators of humoral immunity whereas T lymphocytes

mediate cellular immunity through activation of T helper (CD4) or Cytotoxic T cells (CD8) (5,40).

Inflammation. While immunity helps to fight off invading pathogens in the body, inflammation is part of the immune response that helps to localize the necessary cells and proteins. In response to a reaction, injury, or infection, inflammation occurs with the appearance of the classic four symptoms: edema, redness, heat, and pain. Acute inflammation involves the neutrophil (PMN) cells in comparison to chronic inflammation which involves mononuclear cells (40). Mononuclear cells include macrophages, T lymphocytes, and plasma cells (40) which produce cytokines that mediate inflammation in the acute phase response (49). The acute phase response involves fever, B and T lymphocyte activation, and synthesis of acute phase proteins. The following systemic response is a reparative phase that involves anorexia, leukocytosis, and other metabolic changes. Many of these changes are mediated by the pro-inflammatory cytokines IL-1, TNF-α, and IL-6 (5). These cytokines can trigger other immune cells to secrete more inflammatory cytokines such as interferon-ɣ and interleukin-2 (IL-2) (49).

Although pro-inflammatory cytokines have a beneficial purpose in fighting off infections, high concentrations of circulating inflammatory cytokines are correlated with severe inflammatory conditions such as septic shock and meningococcal septic shock (50,51). In other inflammatory diseases such as rheumatoid arthritis, Eastgate et al.

observed higher concentrations of IL-1 compared to healthy controls which correlated with the inflammatory condition (52). It is based on these observations that dietary interventions with omega-3 PUFAs may have beneficial effects in alleviating chronic inflammatory diseases (49).

The anti-inflammatory cytokines activate the production of eosinophils, mast cells, and immunoglobulin E. Cytokines IL-4, IL-5, and IL-10 are produced by Th2 cells in response to the omega-3 fatty acid composition in plasma membranes (40). The response of cytokine production to typhoid vaccination was investigated before and after 6-8 weeks of fish oil supplementation (1.15-1.56 g/d of EPA and DHA, respectively) in young healthy subjects, showing a reduction of baseline IL-1 and IL-6 levels but not in TNF-α. These results however, were not significant nor were plasma phospholipid fatty acid concentrations measured to assess compliance (53). Additionally, a placebo-controlled parallel-arm trial observed similar effects comparing a high-ALA, low-LA flaxseed oil-based diet to a low-ALA, high-ALA sunflower-oil based diet both supplemented with fish oil (1.6 g/d of EPA and 1.1 g/d of DHA) for 4 weeks after 4 weeks of modified diets. The authors observed a significant inverse relation between EPA content and TNF-α and IL-1β production, with EPA concentrations approximately 1% of phospholipid fatty acids. These results support the conclusion that n-3 fatty acids suppress cytokine TNF-α and IL-1β production (54).

Nutritional Effects on Immune System. Vitamins and minerals have a profound effect on the immune system whether they are present in excessive or deficient amounts. The nutritional status of individuals needs to be balanced and adequate in order for the immune system to function effectively and optimally. The following nutrients have the most profound effect on immunity: dietary fatty acids, vitamins A, B6, B12, C, E, zinc, and iron. Multiple nutrient deficiencies negatively affect the immune system more than single-nutrient deficiencies (40).

Vitamin A status has been extensively studied in association with childhood morbidity and mortality since the early 1930's (55). The Ghana VAST Study Team conducted a double-blind, randomized, placebo-controlled trial with 21,906 children in northern Ghana from 1989 to 1991. Children were randomly assigned to groups receiving either 200,000 IU retinol equivalents every 4 months or placebos of peanut oil capsules. The researchers found that mortality rates due to acute gastroenteritis were significantly lower in the vitamin A supplement group than the placebo group ($p=0.02$). In addition, all other mortality causes were lower in the vitamin A group than placebo group but not achieving statistical significance (56). Vitamin A maintains epithelium integrity in the respiratory and gastrointestinal tracts, thus a deficiency can increase the risk of infections and relates to 1.2-3 million deaths per year (57).

A group of researchers at the Harvard School of Public Health analyzed the effect of vitamin A supplementation in children with viral

gastroenteritis living in Mexico. In a 10-month trial conducted in 1998, 127 children less than 2 years of age were enrolled and given a vitamin A treatment solution every 2 months (20,000 IU retinol for children younger than 12 months of age or 45,000 IU retinol for children 1 year or older). Stool samples were collected at least 3 times a month to detect norovirus infection. Children in the vitamin A supplement group had significantly less ($p=0.01$) norovirus-associated diarrheal episodes compared to the placebo group. These findings indicate the reduced gastrointestinal inflammatory response with vitamin A supplementation inhibit the dominant Th1 response to norovirus infection, thus contributing to lower rates and duration of diarrheal symptoms (58).

Nutritional supplementation with vitamin D has been observed with improved immune response in viral infections with seasonal influenza A. In 2009, Urashima et al. conducted a 4 month randomized, double-blind, placebo-controlled, parallel-arm trial which analyzed 334 children ages 6-15 years, given 6 tablets daily (1200 IU Vitamin D3 or placebo) during the winter months. The incidence of influenza A in children receiving vitamin D3 (18 of 167 subjects) was significantly less than children receiving the placebo (31 of 167; $p=0.04$). The investigators reported vitamin D3 supplementation may improve innate immunity by enhancing antimicrobial peptides and lower inflammation by reducing cytokine secretion (59). These nutritional supplement investigations are just a glance into the benefits nutritional status' show in the immune system.

Upper Respiratory Tract Infections

Physiology. The respiratory system is made up of organs that are involved with the mechanical process of breathing, taking oxygen in (inhalation) and expelling carbon dioxide out (exhalation). Air travels from the nose all the way to the capillaries in the lungs. Following the pathway, air enters through the nose, the nasal cavity, the pharynx (throat), the larynx (voice box), and the trachea (windpipe), which divides into two bronchial tubes that lead into separate lungs. Each bronchus separates further into bronchioles, which terminate into air sacs called alveoli. Capillaries in the lungs surround the alveoli where the final exchange of gases take place (60).

The process of respiration takes place at two levels occurring simultaneously within the body. External respiration is the exchange of gases at the lungs whereas internal respiration involves the exchange of gases at the cellular level within all body organs. In external respiration, the lungs take in oxygen from an inhalation which passes into the capillaries surrounding the alveoli, entering the bloodstream. At the same time, carbon dioxide, a gas by-product from cellular metabolism, travels from the capillaries into the lungs to be exhaled. With internal respiration, oxygen travels from the bloodstream into individual body cells in exchange for carbon dioxide which travels through the bloodstream to the lungs for exhalation (60).

The respiratory tract is characterized by a large surface area continually exposed to the external environment which cycles about 10 liters of air each minute. This feature makes the respiratory tract one of the more frequent sites for infections than any other organ (61), therefore multiple innate and adaptive immune defense mechanisms exist for effective antiviral responses (62). In addition to altering an immune response, the nasal cavity has functional defensive roles for trapping large particles in the ciliated mucosa (63) or signaling the contraction of respiratory muscles to cough or sneeze when irritants are sensed in the airways (61). Other functions include the process of air-conditioning which warms and moistens the air similar to alveolar conditions and olfaction which uses smelling sensors located at the bridge of the nasal cavity (63).

Prevalence & Transmission of URTIs. Upper respiratory tract infections (URTIs) affects ~ 25 million Americans to visit their primary care physicians each year (64). The common cold virus is implicated in 22 million days of work absences and 20 million days of school absences each year, according to the National Center of Health Statistics (65). Due to these lost productivity days it is not surprising that the common cold attributes to serious economic burdens as well as increased morbidity in modern society (66). In the United States, the estimated annual costs of acute respiratory illnesses is $40 billion which puts respiratory infections as one of the top 10 most costly illnesses (67).

The highest incidences of the common cold appear seasonally. In temperate regions of the northern hemisphere, URTIs rise in the autumn, remain high throughout winter, and then decrease in the spring (68). On average, incidences of the common cold appear more often in children than adults. Children usually have 2-6 cold episodes per year whereas adults average 1-3 episodes per year (65). A 2005 cohort study reported the incidences of URTIs and their effect on general health and academic and work performance in 3,249 college adults. Out of those surveyed, 91% of students reported having at least 1 cold, and 4,263 days of missed class were a result of having a URTI (69).

Transmission of respiratory viruses likely occurs from direct or indirect contact with surfaces that have the cold virus on them, then touching the eyes or nose. Infection can also occur from inhaling mucus droplets suspended in the air filled with the cold virus (65).

Infection & Treatment. Although there are over 200 different viruses involved in the common cold, the rhinovirus is the most common comprising 30-50% of all cold cases each year (65,66). The primary route of infection with the rhinovirus in humans is through the eye or the anterior nasal mucosa. The rhinovirus then travels to the posterior nasopharynx, binds to specific receptors on the endothelial cells where the virus enters to start replicating rapidly. The incubation period for rhinovirus ranges from 1 to 7 days although the type of virus and the health status of the host are both factors impacting cold severity (68). In general though, cold

symptoms may last from 7 to 14 days with most people recovering within 10 days (65).

Common treatments of URTIs range from herbal supplements such as Echinacea, nutritional supplements of vitamin C and zinc, and antiviral therapies. A recent study from Johnston et al. observed a significant reduction of self-reported cold symptoms among college males consuming 1000 mg of vitamin C daily compared to controls (70). In studying the same variables, a larger randomized, double-blind, population-based study observed the relationship between the incidence of common colds and vitamin C supplementation. Two intervention groups were analyzed as a high-dose (500 mg/day; n=124) group and a low-dose (50 mg/day; n=120) group for 5 years. The high-dose vitamin C group experienced significantly fewer incidences of colds compared with the low-dose group (71). These studies provide evidence that vitamin C supplementation protects against the incidence of cold symptoms.

The Echinacea plant has been commonly used in the treatment and prevention of URTIs and other infections (66). However, the reliability of research studies and the variable phytochemical compositions of different Echinacea species make it difficult in ascertaining conclusive results (66,72). A community-based study analyzed the efficacy of a dried encapsulated Echinacea treatment in subjects experiencing current cold symptoms and no significant differences were seen between groups (73).

In a similar study design, a randomized, double-blind, placebo-controlled trial tested the efficacy of Echinilin, a standardized Echinacea capsule. Unlike the previous report, this trial used a preparation from freshly harvested Echinacea plants composed of alkamides (0.25 mg/mL), cichoric acid (2.5 mg/mL), and polysaccharides (25.5 mg/L). Researchers found total symptom scores in the Echinacea group to be 23.1% significantly lower than the placebo group ($p<0.01$) (74). Mixed results in Echinacea studies may be attributable the variable active phytochemical composition, thus more research is needed in this area. On the other hand, antiviral drugs may have some beneficial effects but they may have adverse side effects and could potentially bring about viral resistance (75).

Zinc supplement studies treating URTIs in areas where malnutrition is a public health concern, have shown promising effects. A double-blind, placebo-controlled 5-month trial observed significantly fewer common cold episodes per child (1.37 ± 0.86) in a zinc-supplemented group (10 mg/d, 6 d/wk) compared with children in the placebo group (3.15 ± 0.55) ($p<0.001$). Although specific mechanisms of how zinc mediates the immune response were not discussed, this trial supports the importance of zinc for overall immune function in humans (76).

Investigations with other nutritional supplements such as vitamin E may also improve the immune response. A randomized controlled trial in 2004 investigated vitamin E supplementation and respiratory tract infections among elderly nursing home residents. From 1998 to 2001, 451

people completed the 1-year study and were administered a vitamin E (200 IU) capsule or placebo capsule daily. Outcome measures included incidence of, number of people with, and number of days with upper and lower respiratory tract infections. The two groups did not differ statistically in the incidence or duration of respiratory tract infections, however a post hoc analysis identified fewer people in the vitamin E group contracted 1 or more respiratory tract infectionsx with the common cold being the most frequent. These results suggest that vitamin E may have protective effects against common colds more so than any other respiratory tract infection (77).

Immune System & URTIs. A compromised immune system may allow a URTI to persist. For example, prolonged high intensity exercise may suppress the immune response through exercise-induced changes in stress hormones and cytokine concentrations, (78) increasing the risk of a URTI (79). Physical activity may lower natural killer cells, T lymphocytes, nasal neutrophil phagocytosis, and mucociliary clearance, all of which could increase susceptibility to URTIs (78). Although there are some reports that indicate moderate levels of physical activity may decrease the risks of respiratory infections. A recent 12-week study by Nieman et al. observed the effects of regular physical activity with URTI frequency and severity, measured using the reliable WURSS-21 survey. Overall, total days with URTI were 43% lower in those who perceived themselves as highly physical fit and 46% lower in those who participated in aerobic

activity daily. The severity symptoms were reduced 32-41%, respectively (80). More evidence using a reliable and valid URTI measures, such as the WURSS, are needed to understand the relationship between URTI incidence and physical activity levels.

Psychological stress may also contribute to immune dysregulation, affecting components of the innate and adaptive immune responses. The hormone cortisol acts as an indicator of mental stress levels, and some studies suggest sleep deprivation restricted to 4 hours per night markedly increase cortisol levels higher than sleep restricted to 8 and 12 hours per night (81). It is no doubt the type of psychological stress imposed and the duration have varying immune dysregularities, which in turn affect ones risk for infection (82). However, healthy humans have immune systems that are resilient to even large changes in stress-induced immune dysfunction, inhibiting susceptibility to infection. The exception is for individuals already in a disease-state such as with autoimmune diseases, the immune system is inflexible leading to increased vulnerability to infections with stress-induced immune responses (83).

Fish Oils and URIs

Immunosuppressive. The most notable immunosuppressive effects of dietary n-3 PUFAs have been observed in epidemiological studies of the Eskimo population. Large amounts of omega-3 PUFAs are consumed in Eskimo populations with decreased incidences of inflammatory

diseases compared to Americans (6). Although there appears to be fewer incidences of inflammatory diseases, in the past the Inuit population had reports of increased incidence of tuberculosis (84). Based on the multifactorial influences of genetics and environmental exposure including diet, it is difficult to ascertain whether dietary omega-3 fatty acids are related to these incidences of infectious diseases (6).

Animal studies have extensively studied the effects of FO supplementation with infectious disease states. Negative outcomes of increased mortality were seen in mice fed high FO diets after infection with *Salmonella typhimurium* compared to coconut oil (CO) fed mice (85). Conversely, Blok et al. observed improved survival in FO-fed mice infected with *Klebsiella pneumoniae* compared to palm and corn oil controls after 6 weeks of supplementation. The amount of oil comprised 15% of their diet and showed more significant immune-protective effects in FO-fed mice with the lowest infectious dose (86). These variable effects with n-3 fatty acids may be reflected in the study designs of supplement duration, type of infectious organism, or the amount of diet consumed in animal experiments assessing survival rates.

The immunosuppressive effects of n-3 fatty acids in humans has varied and diverse support for several inflammatory conditions. Most studies have observed the effects in patients already in an inflammatory state, not taking into account that each disease differs in severity and in response to inflammatory mediators. The difficulty in measuring the

immunosuppressive effects of n-3 fatty acids involves the subjects' prior health status and the amount and type of n-3 PUFA, which should all be taken into consideration.

Decreases Lymphocyte Proliferation. There are numerous studies examining the effect of dietary n-3 fatty acids on immune cell function, specifically measuring lymphocyte proliferation. T lymphocytes are a measure of the immune response such as when the white blood cells start proliferation it indicates a heightened and alerted immune system. There are several studies that examine FO and its effects on the immune response.

Supplementing n-3 fish oils (1.23 g/d of EPA and DHA) to low-fat diets without additional vitamin E for 24 weeks in elderly men and women significantly reduced lymphocyte proliferation and in vitro production of IL-1β, IL-6, and TNF-α pro-inflammatory cytokines. Conversely, the low-fat, low-fish diet supplemented with vegetarian sources of n-3 fatty acids (0.27 g/d of EPA and DHA) showed moderate increase of these same immune response indexes (87). These data suggest that the immune and anti-inflammatory effects of n-3 rich diets need to be taken into consideration when making recommendations based on population-based studies.

In a 2001 study by Thies et al., 46 healthy older adults aged 56-74 were randomized into different fatty acid treatment groups, supplementing with a placebo of palm and sunflower seed oil blend, ALA, GLA, AA, DHA,

or FO (720 mg EPA and 280 mg DHA) for 12 weeks. Total fat intake from these capsules was 4 g per day. This was the first study to observe effects on the human immune response with a low dose of FO (1 g/d). They did not observe significant influences of n-6 or n-3 PUFAs on inflammatory cell numbers or ex vivo production of inflammatory cytokines. However, significant reductions in plasma soluble adhesion molecule concentrations was observed in the FO and ALA supplement groups compared to changes seen in the placebo group, which contributes to the reported health benefits of n-3 PUFAs. These results suggest that moderate increases of n-3 and n-6 PUFAs may not compromise immune function in healthy older adults (15).

Age may play a factor in the suppression of T lymphocyte proliferation with supplementation of 2.4 g of EPA + DHA with 4 mg of vitamin E for 3 months. A significant reduction of T lymphocyte proliferation was observed in women aged 51-68 years and young women aged 23-33 years, although greater reductions were seen in older women. This is attributable to higher concentrations of EPA and DHA in plasma membranes of older women than in younger women (10-fold increase vs 5-fold increase in EPA and 2.5-fold increase vs 1.6-fold increase in DHA) (88). The clinical implications suggest the anti-inflammatory effects of n-3 fatty acids may not be desirable in healthy older individuals because of the suppression of the cell-mediated immune response observed.

As stated previously, nutrients especially vitamin E have been observed to play a significant role in the immune response. The studies that have not observed significant T cell inhibition with n-3 supplementation involved an additional supplementation with a high dose of vitamin E (205 mg of α-tocopherol) (89). Another factor that may play a role in lymphocyte function may be the type of fatty acid supplemented. Some studies have observed no effect on T lymphocyte proliferation when supplementing with only DHA at 6 g per day for 12 weeks (90). This suggests DHA may have selective effects on immune response because although lymphocytes were unchanged, the production of pro-inflammatory cytokines IL-1β and TNF-α were inhibited and NK cell activity was also significantly reduced after 3 months in a repeat study (91). In analyzing the roles of other n-3 fatty acids, some indices of an altered cell-mediated immune response were observed. When ALA is supplemented via flax-seed oil diets for 8 weeks, the in vitro proliferation of peripheral blood mononuclear cells was significantly reduced compared to controls although no affect in IL-2 secretion or in B cell function (humoral immunity) was observed (92). Purified esters of EPA were analyzed exclusively without the addition of DHA, and significant inhibition of NK-cell activity of peripheral blood lymphocytes was observed with emulsions containing 0.3 g of EPA (93). Although clinical implications are inconclusive, these studies indicate great variability in suppression of immune indices with differing omega-3 fatty acids.

Immune Response Against Infections. In order to defend against pathogens, the innate and adaptive immune responses must operate quickly to induce cellular lymphocyte activity. In order for a sufficient immune response to occur, the number and functions of lymphocytes is important. Several experimental studies in humans have examined the immune response to infections with rhinovirus (RV). Increased peripheral blood mononuclear cell (PBMC) proliferation, prior to RV-16 inoculation, was associated with reduced peak viral shedding ($r=-0.62$, $p<0.005$), thus infections were reduced. Similar findings were observed with RV-induced IFN-ɣ secretion, which was associated with reduced peak viral shedding ($r=-0.58$, $p=0.01$). This confirms the importance of lymphocyte proliferation for sufficient immune responses to RV infections (94).

In considering the health state of the individual, lymphocyte proliferation may be problematic in conditions where the inflammatory response is already heightened. A study by Skoner et al. observed the effects on several immune parameters to RV-39 infections in patients with allergic rhinitis (AR) and nonallergic patients. AR patients showed immune dysregulation with a lower number of activated helper T cells and poor lymphocyte response to RV-39 infection (95). This is evidence that pre-existing inflammation may alter the ability to fight infections related to immune dysregulation.

In order to elicit an immune response, T cells depend on dendritic cells (DC) for effective antigen presentation. Also, DCs produce TNF-α

which stimulates DC maturation to initiate a series of changes that produce a T cell response against infections. Immune function against influenza virus infection was assessed among healthy older adults compared to healthy young adults in an experimental trial. Findings showed that older adults had impaired DCs from a dysregulation of influenza-specific CD8+ T cells. This is evident from observations of decreased proliferation and significantly less TNF-α production compared to young adults. DC maturation is essential for a protective immune response against viruses and is dependent on the production of TNF-α by DCs in older adults (96).

The previous investigations have indicated the need of lymphocyte proliferation for an effective immune response against infections. However, n-3 PUFAs have been shown to decrease lymphocyte proliferation. Fish oil supplementation, rich in n-3 FAs, may interfere with the immune response to the the rhinovirus infection.

Cytokine production. A number of studies have looked at the effects of fish oil supplementation on the production of cytokines IL-1β and TNF-α. In studies supplementing at least 2.4 g or greater of EPA and DHA per day in diets of young adults for 4 weeks reported a 25% to 75% decrease of in vitro secretion of these cytokines (52,88,89). As reported previously, Meydani et al. found a reduction of inflammatory IL-1β, TNF-α, and IL-6 cytokine secretion by 24, 40, and 46% respectively in adults over the age of 40 consuming a low-fat, high-fish diet (1.2 g of EPA and DHA per day)

for 24 weeks. Conversely, the low-fat and low-fish diet (0.27 g of EPA and DHA per day) group modestly increased production of IL-1β and TNF-α secretion but had no effect in IL-6 secretion (87).

The anti-inflammatory effects of omega-3 supplementation with at least 2.3 g of EPA plus DHA per day showed a reduction of the inflammatory cytokines TNF-α, IL-1, IL-2, IL-6, and IFN-ɣ secreted from mononuclear cells (97). However, there is a large discrepancy in the immunomodulatory effects of omega-3 PUFAs in human immune function with many studies varying in the amount of fish oil supplemented from modest to high doses. The reason for such conflicting results of the immunosuppressive effects of omega-3 fatty acids may be due to study design, cytokine measurement methods, and differing subject characteristics (5). More careful analyses of the results of these studies should be taken into consideration.

Current Evidence. The immunosuppressive effects of omega-3 fatty acids have been extensively observed in the human population in regards to lymphocyte proliferation and cytokine production. These measured immune responses are vital in addressing the anti-inflammatory nature of omega-3 PUFAs however, the immune response to infections in healthy adults has not been evaluated. There are several studies addressing this issue in animals suggesting that the immunosuppressive nature of fish oils EPA and DHA may be detrimental to the health of individuals exposed to viral infections (10-12).

Byleveld et al. demonstrated that fish oil consumption in mice impaired host resistance to infection with influenza (11). For fourteen days mice were fed either the control diet of 3 g of sunflower oil plus beef tallow per 100 g of diet or the experimental diet of 17 g of FO per 100 g of diet before being inoculated with the live influenza virus. These levels of FO are comparable to amounts consumed by supplementing adults on a per weight basis. After 5 days of infection, the FO group had significantly greater viral load, lowered body weights, and consumed less food compared to the control group. Other significant observations of immune indices in the FO group were observed such as impaired production of lung IFN-ɣ cytokine, serum immunoglobulin (Ig) G and lung Ig A-specific antibodies; however, lung IFN-α/β and CD4 and CD8 T lymphocytes did not significantly differ between groups. These results suggest that FO may not affect IFN-α/β in the first line of defense but can affect IFN-ɣ once T cells are presented to the antigen (11).

In a similar study as stated previously, Byleveld et al. also examined the effects of FO diets on cytotoxicity and lymphocyte proliferation (10). For 14 days mice were fed either 17 g of FO or beef tallow with 3 g of sunflower oil per 100 g of diet before infection with the influenza virus. It was found that lung virus-specific T lymphocytes were significantly lower in FO fed mice although bronchial lymph node cell proliferation was significantly higher. In addition, it was found that spleen cell proliferation was significantly higher in the FO fed mice. The authors

believe that these results help to explain the delayed viral clearance that was found in FO fed mice (10).

Other researchers examined dietary fish oils and their immunosuppressive effects in response to influenza virus infection. Schwerbrock et al. studied mice fed diets of 4 g of FO plus 1 g of corn oil per 100 g of diet or a control diet of 5 g of corn oil per 100 g of diet for 2 weeks. The FO fed mice had lower lung inflammation compared to controls, but they experienced a 40% higher mortality rate and a 70% higher lung virus load observed as well. FO fed mice also had a longer recovery period after infection. Similar to Bylevleld's results (10), splenic natural killer (NK) cell activity was impaired in the FO fed mice but lung NK activity was not altered. The FO fed mice also had significantly lower CD8 T lymphocytes and reduced mRNA expression of inflammatory protein-1-α,TNF-α, and IL-6 (12).

Based on the significantly suppressed immune responses in influenza-infected mice, a detrimental effect of fish oils might be the contributing factor to the results discussed above.

Chapter 3

METHODOLOGY

Subjects & Study Design. Healthy women at Arizona State University between the ages of 18 and 38 were recruited through email announcement (Appendix B) using Arizona State University ListServs and printed fliers. Interested subjects were referred to an online survey (Appendix C) to determine eligibility based on diet, physical activity, medical history, self-reported anthropometric measurements, and ability to meet at Arizona State University's downtown campus on 4 separate occasions. Eligible subjects were contacted to schedule a screening visit (study visit 1).

Exclusion criteria included regular smoking (use of >10 cigarettes per day), BMI >30 or BMI <18.5, regular use of fish oil supplements, regular consumption of one or more 3.5 ounce servings of fish per week, and vegetarians who excluded all meat, fish, and poultry from their diet. We also excluded individuals who used prescription medications that may interfere with body weight such as thyroid medications, and/or regular use of anti-inflammatory corticosteroids or non-steroidal anti-inflammatory drugs. Women who were pregnant or lactating, individuals who had immunity for the seasonal flu, those who had a seafood allergy, as well as competing and/or training athletes who engaged in vigorous exercise more than 5 times per week were also excluded.

To determine a sample size, a power analysis was calculated using a probability of 0.05 and a power of 0.8. An average standard deviation of 25.9 was used based on several parallel studies that measured mean cold symptom changes after administration of a dietary supplement. A verified sample size calculator was used to determine the calculated subjects needed (98). Based on the averages of the calculated sample size and previous studies' sample sizes, a total of 47 participants appeared to be sufficient (Appendix D).

After completing the written consent (Appendix E), baseline anthropometric measurements, including weight, height, and percent body fat, were measured for an accompanying study. Height and weight were measured using a stadiometer and calibrated scale, respectively. BMI and percent body fat were determined using a bioelectrical impedence (Tanita). A brief medical history questionnaire and a validated food frequency questionnaire (FFQ) for dietary omega-3 fatty acids (Appendix F) were also completed after written consent (study visit 1).

Qualifying subjects entering the 8-week trial were then stratified based on age, BMI, weight, and omega-3 fatty acid consumption via FFQ and randomly assigned to either the experimental (FO, fish oil) or control (CO, coconut oil) group. Random assignment to groups was performed by flipping a coin. Supplements were administered using a double-blind procedure. Subjects received either a placebo of CO (1000 mg/softgel) or FO (600 mg EPA + DHA/capsule); both gel capsules were similar in size

and shape and delivered to the subjects in compliance to the double-blind procedure in which only the primary investigator knew the difference. Subjects in the control group were instructed to ingest one softgel of Puritan's Pride Natural Coconut Oil (1000 mg) per day. Subjects in the experimental group were instructed to ingest one capsule of USP-Certified Energy First Omega-3 Fish Oil (400 mg of EPA and 200 mg of DHA/ capsule) per day, preferably with food. Compliance was assessed by capsule counts at weeks 4 and 8 and also by calendar checks (Appendix F). We controlled for the consumption of dietary sources of omega-3 fatty acids by stratifying the subjects based on their responses from the FFQ on how often they consumed foods such as fish, seaweed, flaxseed, walnuts, etc.

At week 1 of the intervention (study visit 2), all subjects received a booklet containing copies of the Wisconsin Upper Respiratory Symptom Survey-21 (Appendix F) to record their illness symptoms and the use of any over-the-counter supplements and/or medications taken to relieve their illness symptoms each day. Copies of the Godin Leisure-Time Exercise Questionnaire to assess weekly physical activity were also included in the booklet (Appendix F). An explanation and practice session took place at week 1 (study visit 2) to ensure proper usage of the questionnaires and to answer any questions from the subjects. A 24-hour dietary recall (Appendix G) was also administered at week 1 to check compliance for eliminating other dietary sources of omega-3 fatty acids

throughout the study. Compliance to the supplements and surveys were checked bi-weekly through email from a blinded Master's list of subjects' first names and emails. Survey booklet compliance was checked at week 4 (study visit 3), and subjects received a new booklet to record symptoms for weeks 5-8. Subjects returned booklets at week 8 (study visit 4). A second 24-hour dietary recall and omega-3 fatty acid FFQ were completed (study visit 4). Fasting blood samples (no food or drink with the exception of water for 8 hours) for the measurement of the inflammatory marker tumor necrosis factor-alpha (TNF-α) were drawn at weeks 1 and 8.

Blood Analysis. Blood marker TNF-α was analyzed using Human Quantikine Immunoassay ELISA kit, which employs the quantitative sandwich enzyme immunoassay technique. Plasma blood samples were collected with EDTA as an anticoagulant. Within 30 minutes of collection, samples were centrifuged at 1000 x g for 15 minutes, plasma was separated and stored at ≤ 20° C until analysis. Samples were analyzed within 7 days of collection (99) (Appendix H).

Wisconsin Upper Respiratory Symptom Survey-21. The Wisconsin Upper Respiratory Symptom Survey (WURSS-21) is a standard and evaluative measure of the symptoms associated with the common cold (Appendix F). The survey consists of 21 questions: a global severity indicator, 19 symptom-severity items measured on a 7-point severity scale, and one question comparing symptom severity to the previous day. In this study we used a modified version of the WURSS-21 which is a

tested, reliable, and valid method for analyzing the incidence, severity, and duration of the common cold (100).

The WURSS-21 assessed symptom severity on a 7-point scale where 1 is considered to be very mild, 3 is mild, 5 is moderate, and 7 is severe. In this study, summing the scores from the first 10 questions provided a measure of sickness severity, summing the scores from the next nine questions provided a measure of functional impairment, and summing all 19 items provided a measure of a global sickness severity.

Statistical Analysis. We performed statistical analysis using the SPSS Statistical Analysis system 20.0 and dietary analysis using Food Processor SQL (version 10.10). Data were reported as means ± standard error (SE). To compare the differences in means of outcome variables we used the two-tailed independent t-test or Chi-Square tests. Nonparametric measures were used if data were not normally distributed. Differences were considered significant at $p \leq 0.05$.

Chapter 4

DATA & RESULTS

Subject recruitment for this trial took place throughout January and February, 2012 at Arizona State University's downtown Phoenix campus. The online survey was completed by 163 prospective subjects, of which only 53 were contacted due to exclusion criteria (***Figure 2***). Out of the 110 participants the major reasons for exclusion were consuming 3.5 oz. servings of fish more than once per week (41%), vegetarian status (14.5%), taking fish oil supplements regularly (7.3%), having immunity for the seasonal flu (7.3%), and a reported BMI >30 (kg/m^2) (6%). After contacting 53 respondents to schedule the screening visit, 18 people did not respond. A total of 35 females were enrolled in the study, stratified by age, BMI, weight, daily n-3 fatty acid intake from FFQ, and randomly assigned to either the experiment (FO) or control (CO) group. This resulted with 18 subjects assigned to the FO group and 17 subjects in the CO group. Throughout the course of the study, 9 subjects were lost to follow-up visits due to canceling week 1 visit (n=1), not showing up to week 1 visit (n=4), scheduling conflicts (n=2), or intention for weight-loss (n=2). The remaining 26 subjects who completed the study were used for data analysis (FO, n=13; CO, n=13). There was 92.8% compliance throughout weeks 1-8 for all subjects taking the pills. Softgel capsules counted at weeks 4 and 8 verified that participants adhered to the protocol, assuming all capsules not counted were consumed.

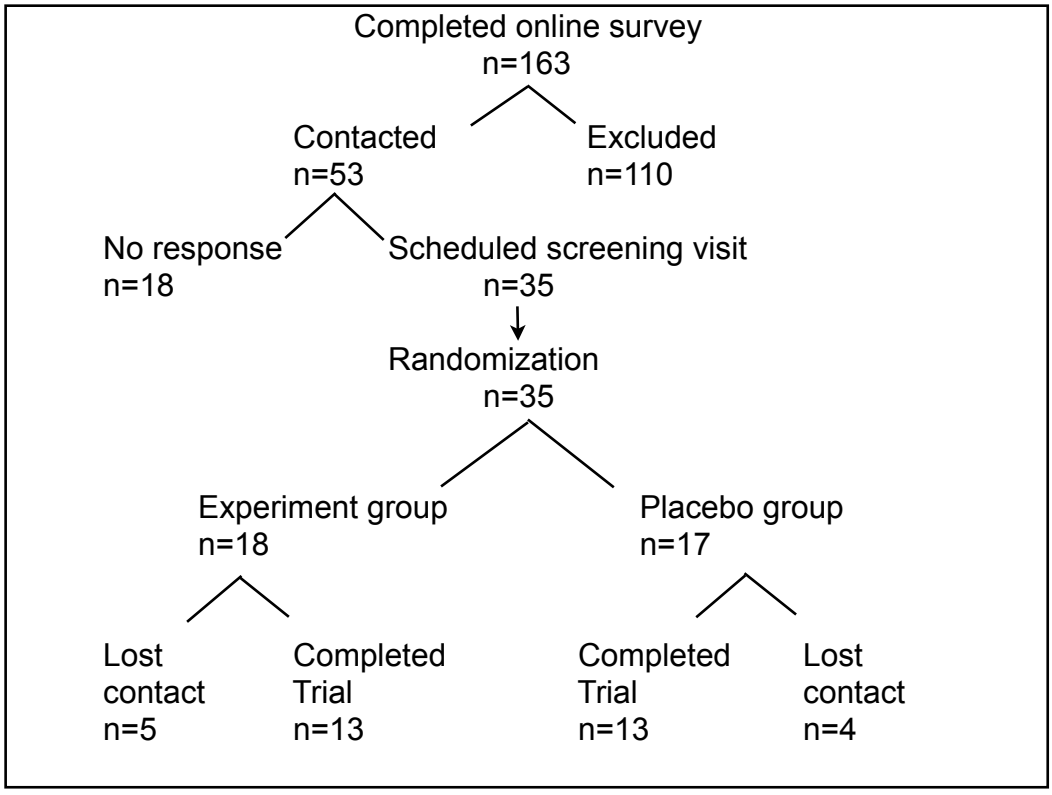

Figure 2. Trial flow chart of subject attrition

Screening variables measured upon enrollment of the study were analyzed using independent samples t-tests, which indicate no significant differences between FO and CO groups at baseline (**Table 1**). Age ranged from 18 to 38 years, with the mean age being similar in both groups (24.0 ± 1.6 years for FO and 23.0 ± 1.7 years for CO). Subjects were stratified on baseline variables of age, weight, BMI, and daily n-3 intake from FFQ. Other variables did not differ significantly between groups, including percent body fat, TNF-α, alpha-linolenic acid (ALA), and linoleic acid (LA) from 24-hour recalls on study visit 2.

Table 1. Baseline Characteristics of Participants[1]				
	ALL (n=26)	FO (n=13)	CO (n=13)	*P*-value
Age[2] (y)	23.5 ± 1.1	24.0 ± 1.6	23.0 ± 1.7	0.339
Weight (lb)	144.2 ± 4.4	140.7 ± 6.1	147.6 ± 6.5	0.424
Height (in)	65.4 ± 0.5	65.3 ± 0.7	65.5 ± 0.6	0.815
BMI (kg/m^2)	23.7± 0.6	23.2 ± 0.8	24.2 ± 0.9	0.424
Body Fat (%)	29.2 ± 1.3	28.4 ± 2.0	30.1 ± 1.8	0.520
TNF-α (pg/mL)	1.9 ± 0.4 (n=19)	2.4 ± 0.7 (n=8)	1.4 ± 0.4 (n=11)	0.205
FFQ n-3 intake[2] (g/d)	0.55 ± 0.08	0.68 ± 0.14	0.43 ± 0.07	0.137
ALA 24-hr recall[2] (g/d)	0.53 ± 0.10	0.50 ± 0.11	0.55 ± 0.18	0.573
LA 24-hr recall[2] (g/d)	4.87 ± 0.91	4.80 ± 1.17	4.95 ± 1.45	0.778
[1]Values reported as means ± SE. ALL - all subjects; FO - fish oil group; CO - coconut oil group; FFQ- food frequency questionnaire; ALA - α-linolenic acid; LA - linoleic acid [2]Data analysis by nonparametric independent samples t-test (Mann-Whitney U); all other analyses by independent t-test				

The subjects were selected based on self-reported low fish consumption, and we tracked ALA (n-3) fatty acid intake at screening (week 0) and at week 8 through a specialized FFQ (**Table 2**). Additionally, 24-hour recall records detailed any changes in LA (n-6) and ALA consumption at week 1 and week 8 among participants. Based on these measures, there were no significant changes in dietary LA and ALA consumption between FO and CO groups over the course of the study.

Table 2. Dietary Data at Weeks 0, 1, and 8[1,2]				
	Week 0	Week 1	Week 8	*P*-value[3]
ALA acid (n-3) intake (g/d), FFQ				
ALL	0.55 ± 0.08	ND	0.60 ± 0.10	0.174
FO	0.68 ± 0.14	ND	0.58 ± 0.14	
CO	0.42 ± 0.07	ND	0.63 ± 0.16	
ALA acid (n-3) intake (g/d), 24-hr recall				
ALL	ND	0.50 ± 0.10	0.55 ± 0.12	0.975
FO	ND	0.48 ± 0.11	0.62 ± 0.21	
CO	ND	0.53 ± 0.17	0.48 ± 0.12	
LA acid (n-6) intake (g/d), 24-hr recall				
ALL	ND	4.83 ± 0.90	4.81 ± 0.84	0.778
FO	ND	4.75 ± 1.15	5.09 ± 1.31	
CO	ND	4.92 ± 1.44	4.53 ± 1.10	
[1]ALA - alpha-linolenic acid; LA - linoleic acid; ALL - all subjects (n=26); FO - fish oil group (n=13), 400 mg/d EPA + 200 mg/d DHA; CO - coconut oil group (n=13), 1000 mg/d coconut oil; ND - no data [2]Values reported as means ± SE; values do not include supplemental fish oil [3]Data analysis by nonparametric independent samples t-test for change over time (Mann Whitney U)				

Due to costs, blood analysis of the plasma inflammatory marker, TNF-α, was performed on a total of 19 randomly selected samples (FO, n=8; CO, n=11). There was a strong correlation with TNF-α concentrations (**Table 3**) for weight (r=0.480, p=0.037) (***Figure 3***), BMI (r=0.481, p=0.037) (***Figure 4***), and percent body fat (r=0.511, p=0.025) (***Figure 5***). The baseline levels of TNF-α did not differ between groups, and the intervention did not change TNF-α concentrations (***Figure 6***). Over the course of the trial, there was not a significant change in TNF-α concentrations seen between CO and FO groups (0.84 pg/mL vs 0.74 pg/mL, p=0.482) (**Table 4**,***Figure 7***).

Table 3. Correlations Among Baseline Variables and TNF-α (pg/mL)[1]		
Variable	Pearson correlation coefficient (r)	P-value[2]
Weight (lb)	0.480	0.037
BMI (kg/m^2)	0.481	0.037
Body Fat (%)	0.511	0.025
[1]Data analysis performed on 19 subjects		
[2]Statistically significant at the 0.05 level		

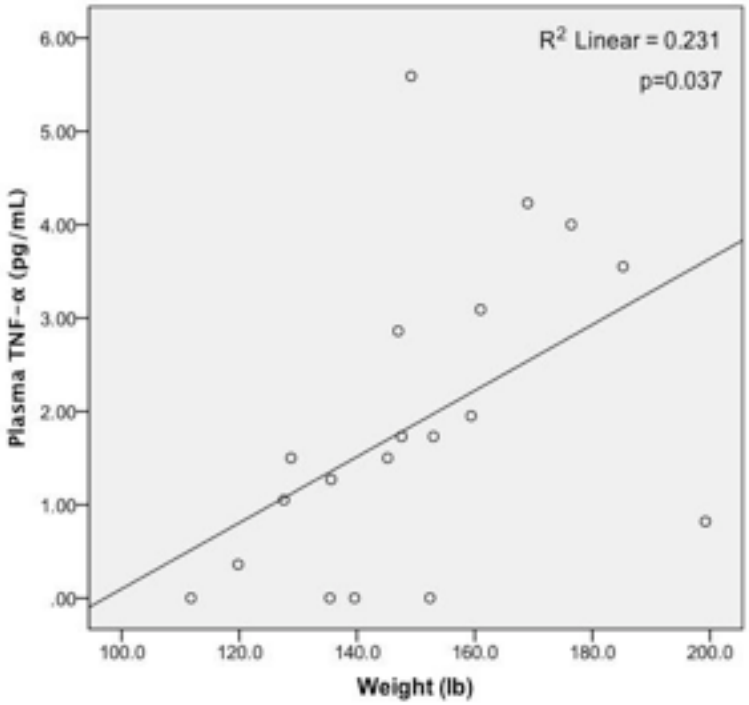

Figure 3. Relationship between baseline TNF-α concentration and body weight

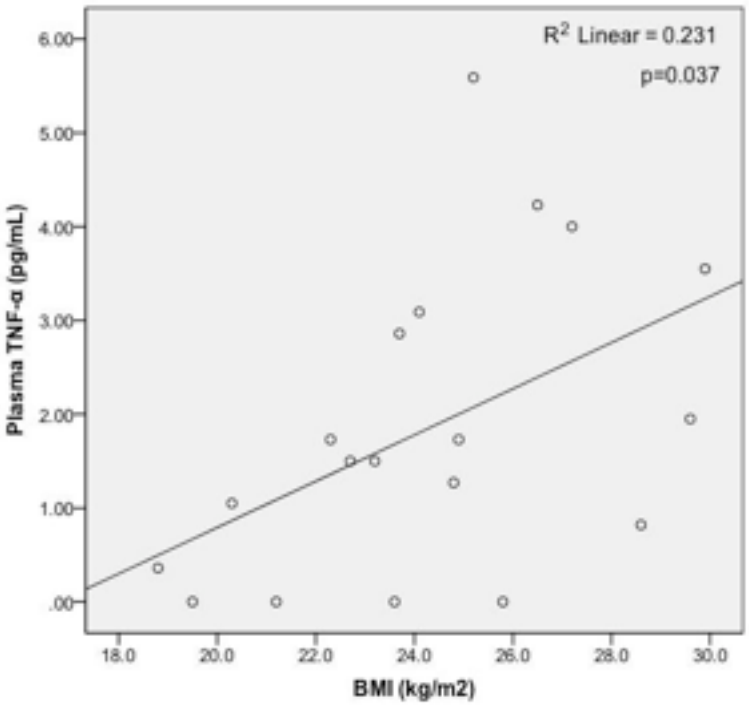

Figure 4. Relationship between baseline TNF-α concentration and BMI

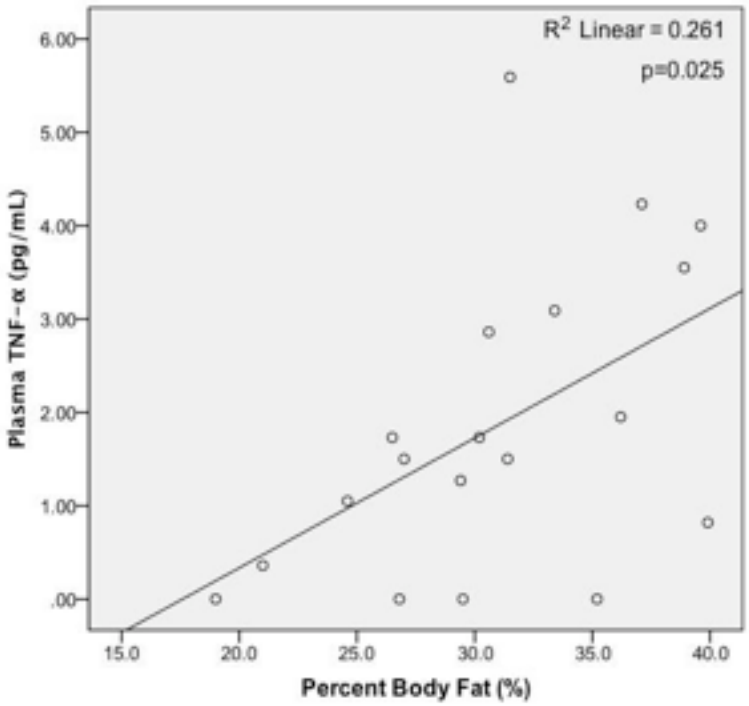

Figure 5. Relationship between baseline TNF-α concentration and body fat percentage

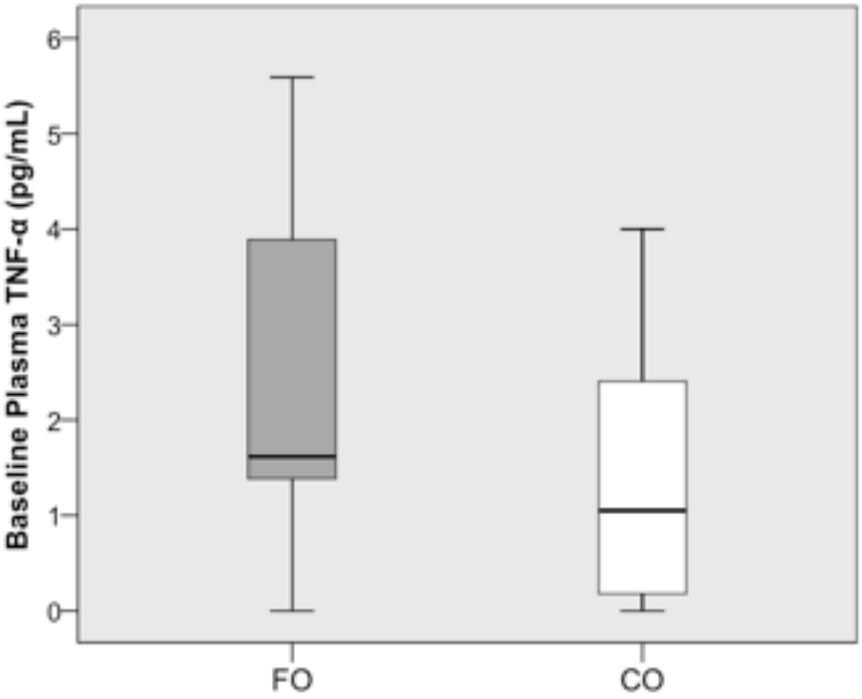

Figure 6. Comparison of baseline TNF-α concentrations between groups
Data did not change from baseline after intervention, no subjects moved across the cut-off point of 1.36 pg/mL (101)

Table 4. Plasma TNF-α Levels (pg/mL) at Weeks 1 and 8[1,2]				
	ALL (n=19)	FO (n=8)	CO (n=11)	*P*-Value[3]
Week 1	1.85 ± 0.37	2.42 ± 0.65	1.44 ± 0.42	0.482
Week 8[3]	2.65 ± 0.71	3.16 ± 1.02	2.28 ± 1.00	
Reported range for healthy controls[4]: 0.60 - 6.40 pg/mL				
[1]ALL - all subjects; FO - fish oil group; CO - coconut oil group [2]Values reported as means + SE; range for entire sample: 0 - 5.59 pg/mL [3]Data analysis by nonparametric independent samples t-test for change over time (Mann-Whitney U) [4]Reported levels of plasma TNF-α from ELISA assays observed in healthy controls (102)				

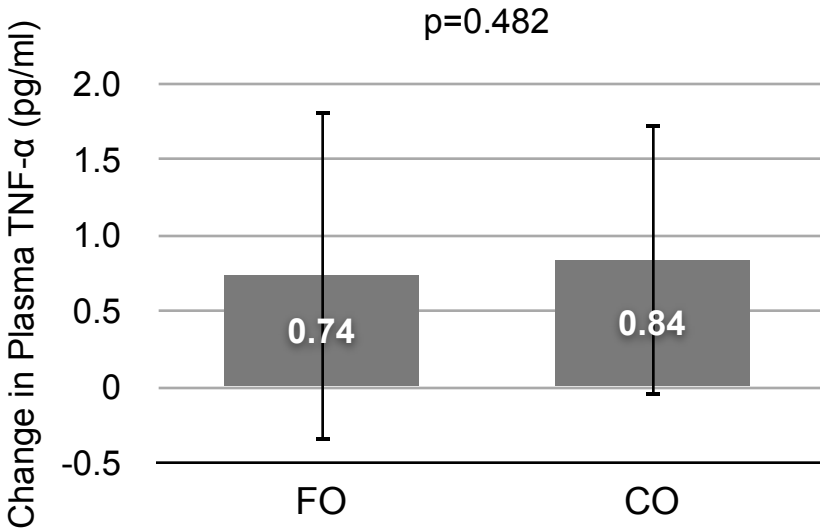

Figure 7. Comparison of changes in mean TNF-α concentrations from week 0 to week 8 between groups

The daily cold symptom survey used to record common cold symptoms during the 8 week trial was the Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) (**Table 5**). The scores are arbitrary units of measure that represent the degree of symptoms. The first 10 questions on the survey assessed common symptoms including runny nose, sneezing, sore throat, cough, head congestion, and fatigue. A 7-point Likert scale was used to indicate the severity of each symptom and a sum of all 56 days calculated the total symptom severity score. The overall compliance for completing the cold symptom survey each day was 98.2% among all participants.

Analysis was performed by taking the average symptom scores over 8 weeks in 4-day increments. No significant differences were observed for all fourteen 4-day averages between groups. Overall, the FO group displayed a lower average perception of symptoms than did the CO

group, but this difference did not achieve statistical significance (p=0.418) (***Figure 8***).

Table 5. WURSS-21 Analysis of 4-Day Mean Cold Severity Symptoms[1,2]

Day	ALL (n=26)	FO (n=13)	CO (n=13)	*P*-value[3]
4	5 ± 2	7 ± 2	4 ± 2	
8	3 ± 2	5 ± 3	2 ± 1	
12	4 ± 2	3 ± 3	4 ± 2	
16	5 ± 2	4 ± 2	6 ± 3	
20	7 ± 3	4 ± 3	10 ± 4	
24	5 ± 2	3 ± 2	7 ± 4	
28	3 ± 1	2 ± 1	4 ± 2	
32	1 ± 1	2 ± 1	1 ± 0.5	
36	2 ± 1	1 ± 1	3 ± 2	
40	2 ± 1	2 ± 2	2 ± 1	
44	1 ± 1	2 ± 2	0.5 ± 0	
48	3 ± 2	5 ± 4	0.5 ± 0	
52	2 ± 1	3 ± 2	1 ± 1	
56	3 ± 2	2 ± 1	5 ± 3	
Total Weeks 1-8	176 ± 44	167 ± 71	185 ± 56	0.418
Weeks 1-4	31 ± 9	27 ± 12	36 ± 13	0.479
Weeks 5-8	15 ± 6	17 ± 11	12 ± 5	0.724

[1]WURSS-21 - Cold symptom survey; ALL - all subjects; FO - fish oil group; CO - coconut oil group
[2]Values reported as means ± SE; Units are arbitrary measure of magnitude
[3]Analysis by nonparametric independent samples t-test (Mann Whitney U)

Analysis of the first and second halves of the trial was performed to assess any changes at midpoint, which is believed to be the time when fish oils are effectively incorporated into plasma membranes. Observed among all participants was a decrease in cold symptom severity scores from the first half (weeks 1-4) to the second half (weeks 5-8) of the trial

(**Table 5; *Figure 8***). However, the CO group decreased more dramatically (67%) than the FO group (37%) from the first half of the study, although not statistically significant (p=0.572).

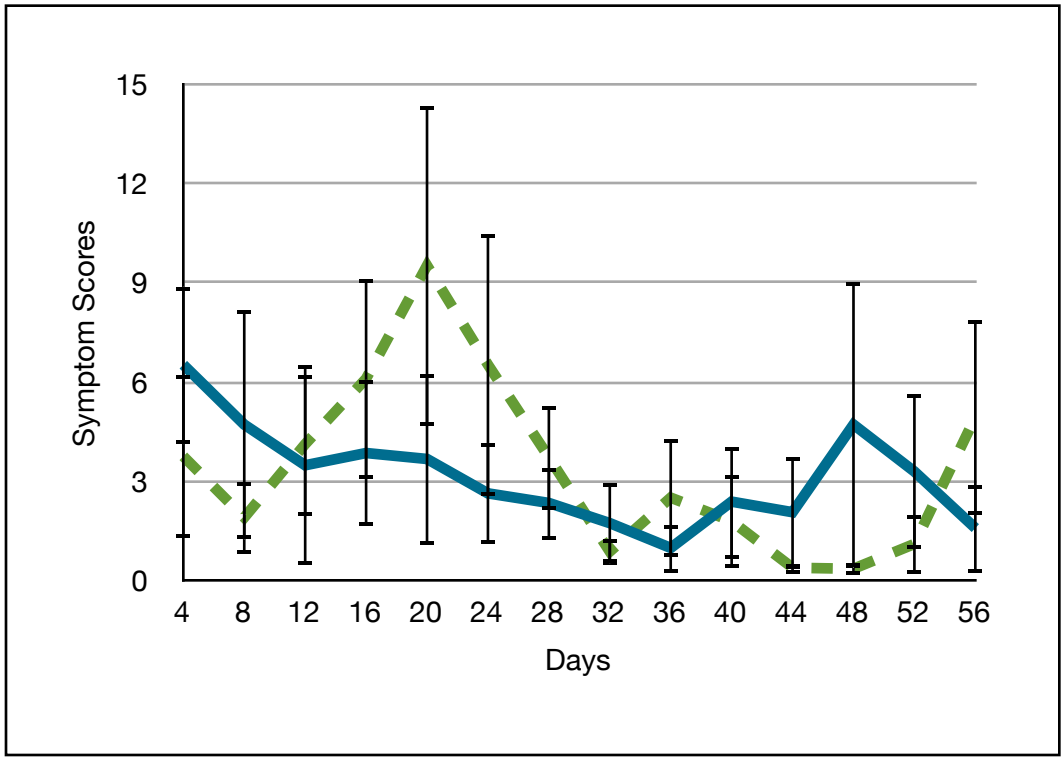

Figure 8. Comparison of mean cold symptom severity scores between groups over course of the study.
Units are an arbitrary measure of magnitude. Symptom scores are reported as 4-day averages (1=very mild; 3=mild; 5=moderate; 7=severe). (FO) (CO)

The WURSS-21 also gave insight into how the subject was feeling in regard to a URTI illness, measured as the global severity indicator. Data were coded as yes or no for each analysis. No significant differences were observed between FO and CO groups in all analyses with the WURSS-21 (***Figure 9***).

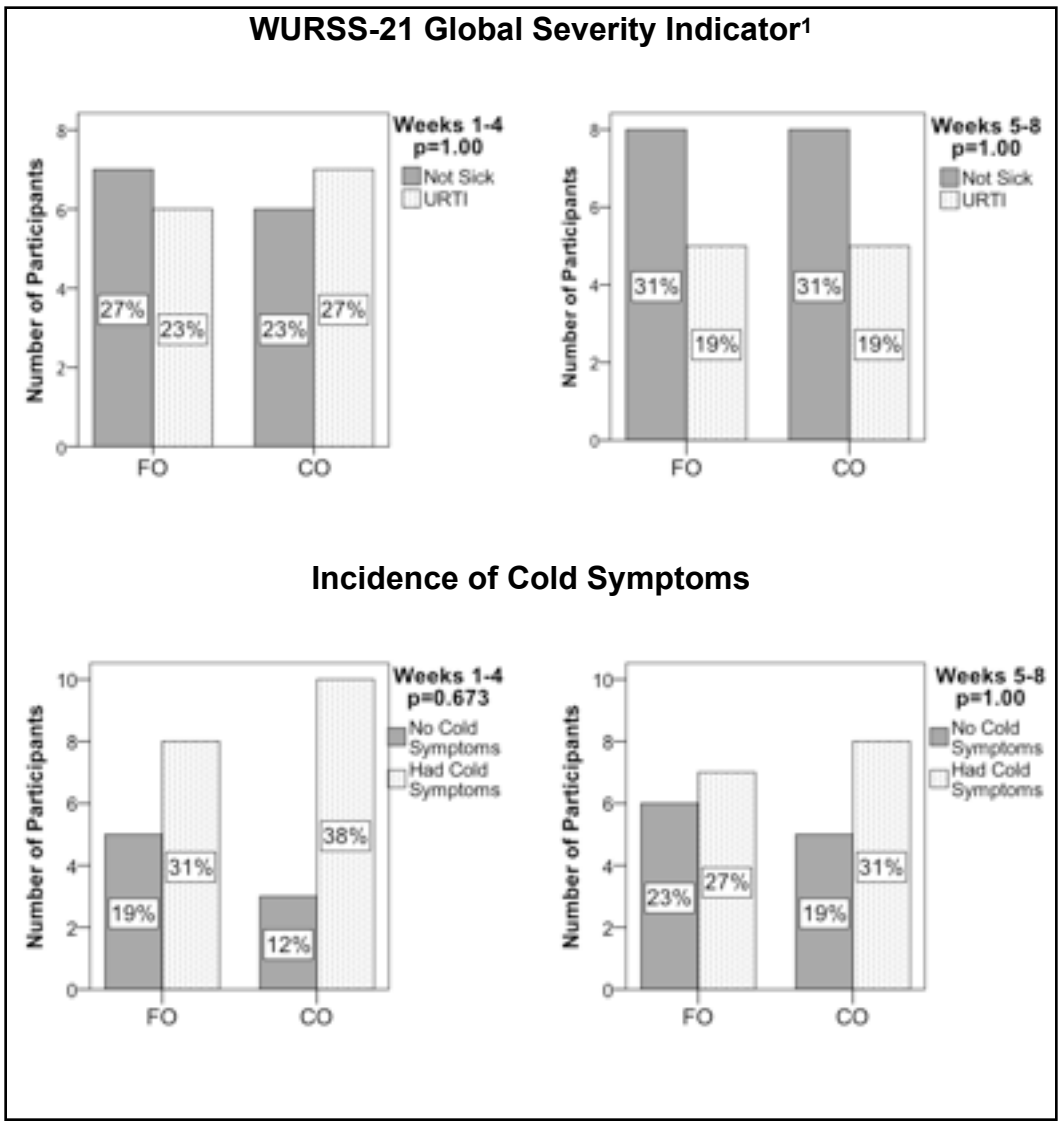

Figure 9. Analysis of WURSS-21 global severity indicator, assessing those not sick or sick with a URTI. Analysis of the incidence of cold symptoms, assessing those with no symptoms or having cold symptoms. Data represent percentages of all subjects (n=26) analyzed between groups from the first (weeks 1-4) and second (weeks 5-8) half of the trial. P-values represent Chi-square (continuity correction values) for 2x2 tables.

[1]WURSS-21 - Cold symptom survey; URTI - Upper respiratory tract infection

To assess the severity of the colds, an analysis of reported cold symptoms greater than or equal to moderate magnitude was performed (***Figure 10***). There was no difference between reporting of symptoms between groups from weeks 1-4 and weeks 5-8. Of those who reported a cold, the incidence of that cold being moderate to severe was also analyzed. No significant differences were observed between groups who reported moderate to severe cold symptoms.

In order to control for other factors interfering with the inflammatory state, reported use of NSAIDs, other medications, and total caffeinated and alcoholic beverages were tracked with yes or no responses to questions added to each daily survey. There were no association of these potential confounding variables with the outcome measures of changes in TNF-α concentration and cold severity symptom scores over time, thus they were not controlled for during data analysis.

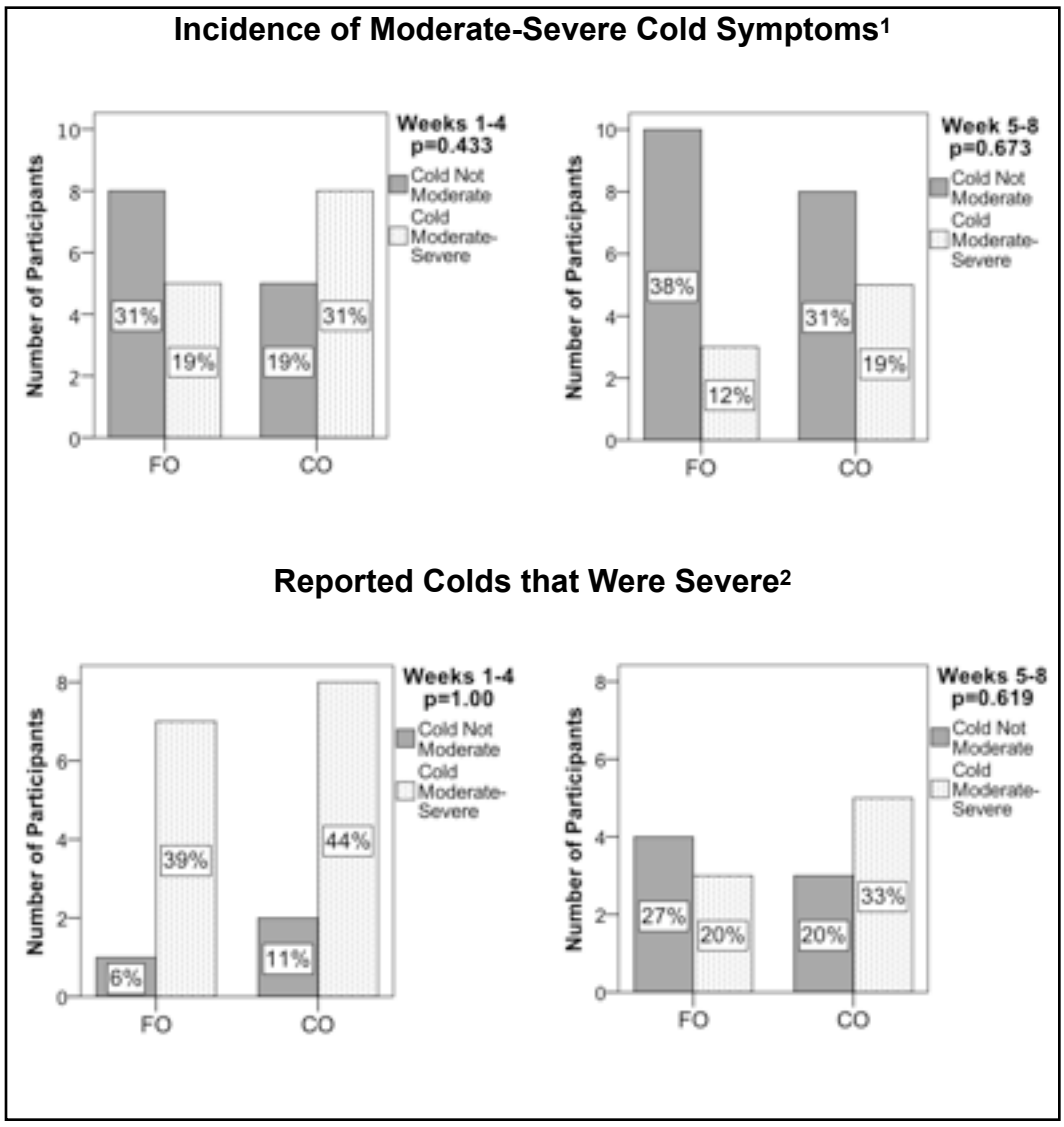

Figure 10. Analysis of the incidence of cold symptoms being moderate or above and reported colds that were severe, assessing subjects' perception of colds being less than moderate and their perception of colds being moderate to severe. Data analyzed between groups from the first (weeks 1-4) and second (weeks 5-8) half of the trial. P-values represent Chi-square (continuity correction values) for 2x2 tables. Error bars show 95% confidence interval.

[1]Data represent percentages of all subjects (n=26)

[2]Data represent percentages of subjects reporting cold symptoms during Weeks 1-4 (n=18) and Weeks 5-8 (n=15)

Chapter 5

DISCUSSION

In this double-blind, placebo-controlled, randomized trial, 8-weeks of FO supplementation (400 mg EPA + 200 mg DHA per day) did not increase the risk for cold infections compared to placebo (1000 mg coconut oil per day). When all data were considered, reporting of cold severity symptoms in CO was 11% greater than FO although these results are not significant. Results from this current study do not negate the American Heart Association's current dietary recommendations to consume 3.5 oz of fish twice per week.

The incidence of symptoms related to URTIs in healthy females consuming 600 mg of fish oil per day does not agree with the infectious risks seen in animal studies. This is the first known study in humans to examine the incidence of cold symptoms with FO supplementation and the data are inconclusive. Overall, it appears the controls had more cold severity symptoms however, looking at the first and second half of the study it does not appear that way. Infected mice showed adverse effects in regard to increased viral load, increased mortality (12), and decreased cytotoxic T cell activity (10,11) while fed FO diets. A longer trial may be necessary to better examine the relationship between FO consumption and infection risk in humans.

It is important to consider the time needed for incorporation of fish oils into plasma phospholipid membranes. A number of placebo-controlled

studies have examined the effects of FO supplementation with EPA + DHA, EPA, or DHA fatty acids on changes of plasma phospholipid concentrations after supplementation. Cao et al. examined healthy men and women consuming 2.16 g (1296 mg EPA + 864 mg DHA) per day for 8 weeks and found a 5% increase in EPA + DHA concentrations in plasma phospholipid membranes, although no statistic significance was observed (40). Rees et al. showed a mean increase of 1.8% in EPA plasma phospholipid concentrations after 12 weeks of supplementation with 1.35 g of EPA per day in young healthy men, which was significantly different than controls ($p<0.02$) (103). Thirdly, Kew et al. studied EPA (4.7 g/d) and DHA (4.9 g/d) supplementation separately for 4 weeks in healthy subjects and showed a mean increase of 5.8% in EPA and 7.5% in DHA concentrations, with each group respectively ($p<0.05$) (104). It is important to consider the amount of EPA and DHA supplemented and the length of the study (4, 8, and 12 weeks), but these studies provide evidence to support the dose-dependent changes of EPA and DHA in plasma phospholipid concentrations after 4 weeks of supplementation.

Thus in the present study, it might be more relevant to account for FO after 4 weeks, which is the second half of the study (Weeks 5-8). However, looking at the data from the first and second half of the trial, there was a larger decrease in cold symptoms in the CO group. The FO group decreased 37% whereas the CO group decreased 67% (**Table 5**), although data are not significant. More research is needed to determine

what effects moderate-dosage of FO supplements have on cold symptoms.

This trial coincided with the common months of cold and flu incidences, however the recent 2011-2012 cold and flu season peaked in February (***Figure 11***). Since the peak of the season was at the beginning of the study, our outcome measure of cold symptoms was on the down-fall. These data are based on the Gallup-Healthways Well-being Index, surveying 1,000 Americans each day if they had a cold or flu yesterday (105). Ideally, the study of cold symptom occurrences should begin in December or January to capture the highest reports of colds among American adults.

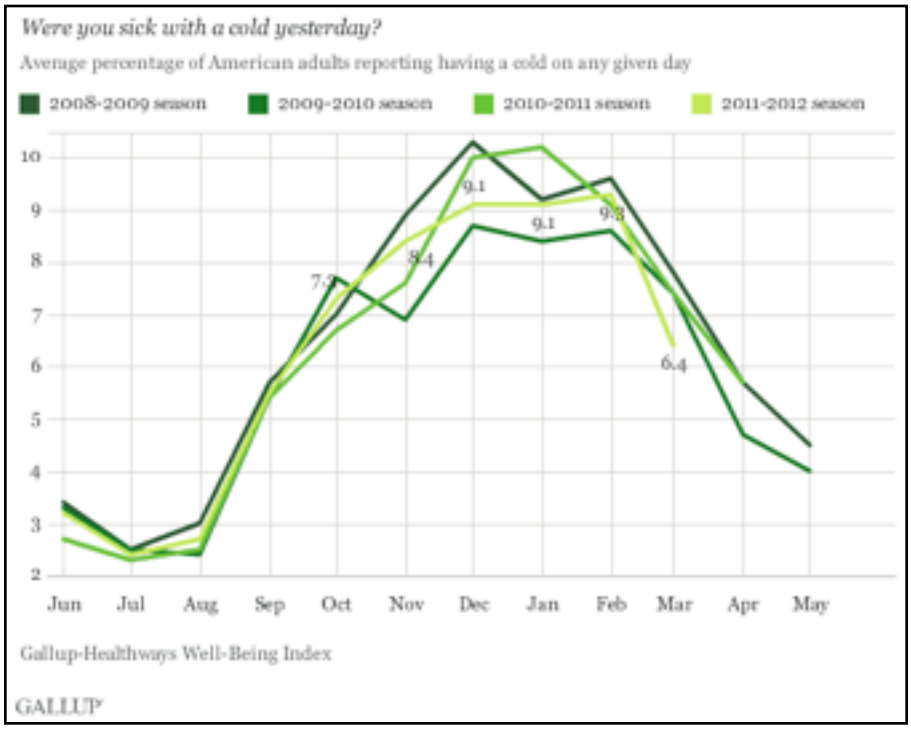

Figure 11. Year-over-year comparison of self-reported cases of colds from the Gallup poll (105).

In our study, baseline TNF-α correlated with body weight, body fat percentage, and BMI. When all variables are considered, it appears TNF-α levels are associated with fat mass in healthy women. Another study measured TNF-α using the same assay and showed a positive correlation with baseline absolute truncal fat (kg) ($r=0.42$, $p=0.01$) and relative truncal fat ($r=0.54$, $p<0.01$), calculated as truncal fat divided by total body fat measured using dual x-ray absorptiometry scans, in healthy young controls, healthy elderly controls, or Type-2 diabetics. After considering compounding factors such as age, gender, and diabetes, TNF-α continued to significantly correlate with relative truncal fat mass ($r=0.830$, $p=0.04$) (106). Further evidence supports that TNF-α levels are associated with visceral fat mass measured using the waist to hip ratio (WHR) in obese women, most of whom also showed some level of glucose intolerance and/or insulin resistance. TNF-α was strongly correlated with WHR ($r=0.53$, $p<0.01$) and fasting and post-oral glucose tolerance tolerance test (OGTT) insulin levels ($r=0.47$, $p<0.02$) (107). However, other observations have associated TNF-α levels in both subcutaneous and visceral fat tissues. Adipocyte cell size in both tissues were significantly correlated with TNF-α in serum and soluble TNF-receptor (sTNFr) 2 production in overweight/obese and lean subgroups (108).

Endogenous production of TNF-α comes from many cell types which includes adipose cells and circulating monocytes and lymphocytes in the blood (106,109). Evidence indicates TNF-α production increases

with increasing adiposity and decreases with decreasing adiposity. TNF-α modulates fat metabolism and controls adipocyte size by possibly increasing insulin resistance in adipose tissue through paracrine signaling (109). Other immune parameters have shown to be elevated in obese individuals, such as increased monocytes (110) which may be attributable to TNF-α production in adipose tissue.

A significant relationship between serum TNF-α concentrations and increasing levels of obesity were observed in women with BMI categories ranging from 25-30 kg/m^2 (6.5 pg/mL, $p<0.001$), 30-40 (6.8 pg/mL, $p<0.001$), and over 40 (7.4 pg/mL, $p<0.001$) compared to lean controls (2.9 pg/mL) (111). Although the relationship is apparent, we did not expect to see anything in our subjects who were screened with a measured BMI < 30 kg/m^2.

Plasma TNF-α concentrations measured in the people with chronic inflammation, such as in patients with Type-2 diabetes, were significantly higher than healthy controls (~2 pg/mL vs. ~1.1 pg/mL, $p<0.05$) (104). This is true for individuals in other chronic inflammatory conditions such as with metabolic syndrome and smoking. Compared to healthy controls, measured levels of serum TNF-α, using similar methods in the present study, in people with metabolic syndrome had 1.45 pg/mL ($p<0.001$) and smokers had 15 pg/mL ($p<0.004$) (101,112). TNF-α was found to be significantly higher in heart disease patients with severe aortic stenosis (AS) (2.1 pg/mL) and mitral regurgitation (MR) (1.3 pg/mL) compared to

healthy controls (0.7 pg/mL) ($p<0.05$). AS patients had significantly higher TNF-α levels than in MR ($p=0.03$) and lastly, patients with AS and MR in New York Heart Association (NYHA) functional class II had higher TNF-α levels and sTNFr 1 and 2 than NYHA class I patients. These findings indicate serum TNF-α levels and sTNFr 1 and 2 are elevated in patients with clinically significant hemodynamic pressure and volume overload (113). A number of factors could contribute to the variability in measured TNF-α concentration such as possible polymorphisms in genes that influence TNF-α production (114), inter-assay or intra-assay variabilities, measuring soluble TNF receptors instead of concentrations, or the medium that TNF-α was measured from such as serum/plasma or cell culture supernates.

Gurrorala et al. determined a cut-off point related to the change in the clinical parameter of high density lipoprotein cholesterol (HDL-c) levels with TNF-α concentration. Significantly higher incidences of TNF-α levels greater or equal to 1.36 pg/mL were observed with HDL-c levels less than 35 mg/dL ($p<0.05$) in those with metabolic syndrome compared to healthy controls (101). The present study observed similar associations with TNF-α and HDL-c levels such that baseline HDL-c had a medium-strength inverse correlation with TNF-α ($r=-0.496$, $p=0.101$), although not significant. At baseline, 58% of all subjects had TNF-α levels above 1.36 pg/mL (***Figure 12***). It is unknown what metabolic conditions could explain this descriptive characteristic since subjects were screened with no

unresolved health issues. Nonetheless, these findings validate the assay that TNF-α is strongly related to body fat and inversely associated with HDL-c levels, which coincides with that reported in the literature (101,106-108).

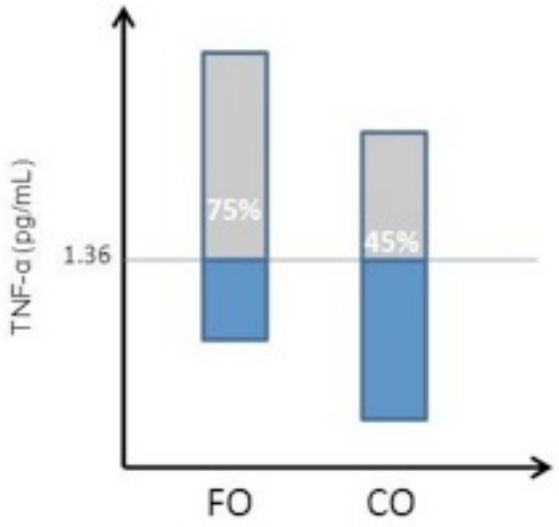

Figure 12. Percentage of participants with TNF-α above the clinical parameter at baseline.
Intervention did not change TNF-α concentrations above or below the clinical parameter 1.36 pg/mL

Future studies should consider measuring plasma concentrations of EPA, DHA, and AA fatty acids at multiple points throughout the trial. This could have effectively monitored compliance to protocol and assess the time-frame for which plasma saturation of fatty acids occurred.

There are gender differences to consider in regard to n-3 PUFA status. The metabolic conversion of essential ALA into DHA is inefficient in humans but more efficient in women (9.1%) compared to men (7.9%) (115). It is believed that estrogen is a mediator in the increased conversion

of ALA to DHA, possibly due to its role increasing the activity of desaturase and elongase enzymes necessary for DHA conversion (116).

Since only healthy women were enrolled for in the trial, this limits the generalizability of the results especially to males, elderly subjects, those with chronic inflammatory conditions, or obese populations. For example, the immune response varies with disease state, age, and body weight therefore it is difficult to generalize these results to other populations.

The placebo of coconut oil supplemented at 1 g/d, was chosen for its trivial effects on metabolic outcomes. It is characteristically high in saturated fatty acids and only 6-12 carbons long, making it a medium-chain triacylglycerol (MCT). MCTs are different than PUFAs in that they do not get incorporated into chylomicrons for transport through the lymph to be delivered to tissues. Conversely, MCTs are transported directly into the cells and metabolized for energy. Other investigators have chosen placebo oils such as mineral oil, soybean oil, corn oil (101) and olive oil (104) because of their limited bioavailability in low doses and high saturated fatty acid content. Studies that used CO to show an effect were supplementing 27.4 g/d that improved health outcomes such as decreased waist circumference and increased HDL (117) or 29 g of coconut oil that showed a decrease in post-prandial glucose and increased total cholesterol levels (118). The effects of 27-29 g/d of coconut

oil reported in these studies are drastically larger doses than the 1 g/d of coconut oil supplemented in the present study.

The WURSS-21 is a validated and reliable measure for assessing the subjective perception of cold symptoms (100). People may tolerate various cold symptoms differently, impacting their self-reported responses on the questionnaire. Since cold indices were measured based on the subject's perception of illness, there is a possibility that cold symptoms could be confused with other ailments different than cold symptoms such as allergies. To avoid confounding symptoms due to allergy, the first question on the survey was modified to specify only sickness related to a URTI. Additionally, daily tracking of any medications taken and the number of caffeinated and alcoholic beverages consumed were accounted for as possibly conflicting with the immune response.

The weekly dosage of EPA and DHA given in supplement form (4.2 g) was equivalent to about four servings of 3.5 oz of oily fish per week. The types of fish that would be equivalent to the amounts given in the trial over the course of a week would be 14 oz of Atlantic salmon, 6.3 oz of mackerel, 10.7 oz of herring, and 15 oz of sardines (119). Based on these results, the dietary intake of fish, at the dosage of twice the recommendations, of approximately 2 oz servings of fish per day, is not problematic. The EnergyFirst fish oil supplement instructions were actually intended for 3 times the amount given in the trial. The health effects from

the instructed supplemental amount of 1.8 g/d EPA + DHA would need to be further investigated.

Limitations. There are several limitations in this trial. Subject adherence to protocol required consuming one supplement and completing the cold symptom survey daily. The subjects were not instructed to abstain from eating fish in order to avoid fish oil. The pre and post FFQs were designed to capture this, thus we are fairly confident of subject adherence. The inflammatory response may be impacted from external factors such as sleep deprivation and psychological stress. The 8-week trial duration may not be long enough to reflect long-term effects of fish oil supplementation. The timing of the trial did not coincide with the peak of the cold and flu season. Although the power analysis suggested 47 participants, only 26 were analyzed due to recruitment restrictions and exclusion criteria which may have hindered observing significant changes. Unlike other fish oil supplemented studies, this study did not observe detailed mechanisms to determine specific changes of immune indexes. This study only measured the incidence of cold symptoms and therefore could not control exposure to cold viruses.

Strengths. The screening of the subjects was performed on a number of exclusion criteria to ensure a homogenous subject pool. Some of the inclusion criteria included non-smokers, consumption of fish less than once per week, not taking fish oil supplements, not taking medications that may interfere with the inflammatory state such as

corticosteroids and/or non-steroidal anti-inflammatory drugs, not having a seafood allergy, and those who did not receive a 2012 seasonal flu shot. The double-blind nature of this trial minimized the placebo-effect and investigator bias, preventing the investigators from knowing which treatment the subjects were receiving.

Conclusion. Moderate doses of fish oil supplementation (0.6 g EPA + DHA per day) for 8 weeks is not problematic for increasing the risk of the common cold infection in healthy females. The TNF-α concentrations displayed a significant positive correlation with body weight, BMI, and percent body fat, providing construct validity to this measure. These findings at the level of fish oil supplemented do not appear to cause unintentional health consequences and do not refute the current dietary recommendations of two 3.5 oz servings per week. However, more research is needed to assess realistic levels of fish oil supplementation (~1.8 g/d EPA and DHA) with the incidence of colds during the prime cold season.

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APPENDIX A

IRB APPROVAL

ASU Arizona State University
Knowledge Enterprise Development

Office of Research Integrity and Assurance

To: Carol Johnston

From: Shannon Ringenbach, Biosci IRB

Date: 01/04/2012

Committee Action: Expedited Approval

Approval Date: 01/04/2012

Review Type: Expedited F2 F7

IRB Protocol #: 1112007220

Study Title: Omega 3 Fatty Acid Supplements and Health

Expiration Date: 01/03/2013

The above-referenced protocol was approved following expedited review by the Institutional Review Board. It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date. You may not continue any research activity beyond the expiration date without approval by the Institutional Review Board.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.
Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.

PO Box 876111 Tempe, AZ 85287-6111 ♠ 660 S. Mill Avenue, Suite 315♠ P 480 965 6788 ♠ F 480 965 7772
http://researchintegrity.asu.edu

APPENDIX B

STUDY RECRUITMENT MATERIALS

Printed Flier:

ASU SUPPLEMENT TRIAL

THE NUTRITION PROGRAM AT ASU IS RECRUITING RESEARCH VOLUNTEERS (18-40 y of age)

HEALTHY YOUNG WOMEN ARE NEEDED

THIS STUDY WILL TEST HOW A COMMON DIETARY SUPPLEMENT AFFECTS HEALTH

Participation will include:

- Traveling to the ASU's Downtown campus to complete the screening process
- Traveling to the ASU's Downtown campus on 3 test days (trial weeks 0,4, and 8 for <30 minutes/visit)
- Consuming a gel capsule daily with meals.
- Fasting 12 hours and restricting exercise the nights before the week 0 and 8 test visits.
- There will be two blood draws on separate days

You will receive a $10 gift certificate at week 4
and a $15 gift certificate at week 8

INTERESTED?? Please visit our recruitment site: https://www.surveymonkey.com/s/asu_supplement_study

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Email Announcement:

Young Women Volunteers Needed for Supplement Study

The Nutrition Program is recruiting healthy young women to consume a dietary supplement for 8 weeks. The study will investigate whether a commonly consumed supplement affects health parameters in women. Participation in this study is voluntary. Participants will receive the supplement and $25 in gift cards during the study.

Individuals who are healthy, 18-40 years of age, willing to consume the commercially purchased gel capsule supplement daily, and are willing to have blood taken on two occasions may be eligible to participate.

For more information or to apply for the study, please visit our recruitment site: https://www.surveymonkey.com/s/asu_supplement_study

APPENDIX C

ONLINE SURVEY SCRIPT

Dietary Supplement Study

https://www.surveymonkey.com/s/QVY9YX7

1. Please provide your email address

2. Please select your gender

- O Male
- O Female

3. Please enter your height and weight

Height (inches)

Weight (pounds)

4. Are you a vegetarian and exclude all meat, fish, and poultry from your diet?

- O Yes
- O No

5. Do you consume any of the following foods more than once per week? (Please check yes or no):

	Yes	No
Eggs	☐	☐
Dairy	☐	☐
Fish	☐	☐
Beef	☐	☐
Flax seed or flax oils	☐	☐
Soy products (e.g., milk, tofu, etc.)	☐	☐

6. Do you take any dietary supplements?

- O Yes
- O No

If yes (please specify)

7. Please check any of the following foods that you are allergic to (check all that apply):

- ☐ Soy
- ☐ Fish
- ☐ Milk
- ☐ Eggs
- ☐ Tree nuts and/or peanuts
- ☐ None of the above

8. Are you pregnant, lactating, or do you anticipate becoming pregnant?

O Yes

O No

9. If you smoke, please select how many cigarettes you smoke per day

O 0

O 1-5

O 6-10

O > 10

10. Do you participate in vigorous, highly intense exercise more than 5 times per week?

O Yes

O No

11. Do you take any of the following medications: e.g. beta-blockers, ACE inhibitors, diphenhydramine or cyproheptadine (allergy medications), lithium carbonate, corticosteroids, insulin, thiazolidinediones (Actos, Avandia, or Avandamet), sulfonylureas, incretins, sodium valproate, or thyroid replacement therapy?

O Yes

O No

12. Will you be able to maintain your current diet and physical activity for a consecutive 8 weeks?

O Yes

O No

13. Are you willing and able to travel to the ASU Downtown Campus to meet with research investigators on four separate occasions?

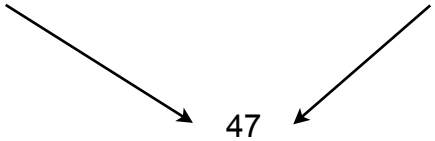
O Yes

O No

APPENDIX D

POWER ANALYSIS

Study	Study Design	N/group	Standard Deviation	Change in Mean	Calculated Subjects Needed
Brown et al. (95)	Parallel	40	1.48	3.53	8
Johnston et al. (68)	Parallel	15	88.5	105	26
Goel et al. (72)	Parallel	54	0.73	0.7	38
Barrett et al. (94)	Parallel	183	13	28	10
Average		73	25.9	34.3	21



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APPENDIX E

INFORMED CONSENT

CONSENT FORM
Dietary Supplementation and Health

INTRODUCTION
The purposes of this form are to provide you (as a prospective research study participant) information that may affect your decision as to whether or not to participate in this research and to record the consent of those who agree to be involved in the study.

RESEARCHERS
Dr. Carol Johnston, Associate Director of the Nutrition Program at Arizona State University, as well as Nutrition graduate students, Megan Gutierrez and Bianca Teran, have invited your participation in a research study.

STUDY PURPOSE
The purpose of the research is to examine the effect of a dietary supplement in adult females, 18-40 years old, on immune function and overall health.

DESCRIPTION OF RESEARCH STUDY
If you decide to participate, then as a study participant you will join a study to evaluate the effect of ingestion of a supplement daily for 8 weeks on health markers. You will be instructed to consume the supplement daily and to complete a one-page questionnaire daily regarding illness. If you are interested in joining the study, you will be asked to come to an initial screening where your height and weight will be measured and you will complete a health history and dietary questionnaire. If you are eligible for the study, you and the other participants will be randomly assigned in either the control (placebo) or experimental (dietary supplement) group. Subjects will be asked to visit the research site on 3 occasions at 0, 4, and 8 weeks. At weeks 0, 4, and 8 you will be weighed. At weeks 0 and 8 a fasting blood sample will be drawn. At each blood sampling approximately 2 tablespoons of blood will be collected. At weeks 4 and 8 you will complete a dietary questionnaire and you will need to bring in your daily survey booklet with your supplement pack.

If you say YES, then your participation will last for 9 weeks at the Downtown Phoenix campus at Arizona State University. Approximately 40 subjects will be participating in this study locally.

RISKS
All gel capsules used in this study have been purchased from retail vendors. There may be a slight chance of gastrointestinal distress (including an aftertaste, burping, and reflux) when taking the supplement on an empty stomach. This risk is reduced if you ingest the capsule with a meal and consume plenty of water. Blood draws may cause light-headedness, headaches, and/or temporary bruising. A research nurse will be performing the blood draws.

BENEFITS
Although there may be no direct benefits to you, the possible benefit of your participation is that you will be able to experience what it is like to be a part of a research study that may provide new evidence to support the health of many adult women.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but the researchers will not identify you. Your name will not be associated with any data related to the study. In order to maintain confidentiality of your records, you will be assigned to a subject
number, which will be used throughout the course of the study to identify you. Only the investigators will have access to subject names and their corresponding codes.

Please initial _____

ASU IRB
Approved
Sign [signature] for Mirianna Ringenbach
Date 1-4-12 to 1-3-13

WITHDRAWAL PRIVILEGE
It is ok for you to say no. Even if you say yes now, you are free to say no later, and withdraw from the study at any time. Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled.

COSTS AND PAYMENTS
The researchers want your decision about participating in the study to be absolutely voluntary, yet they recognize that your participation may pose some costs such as inconvenience and a small time commitment. In order to help defray your costs, you will receive a $10 Target gift card at week 4 and a $15 Target gift card at week 8 visits for a total of $25.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston, Principal Investigator and Professor of Nutrition at ASU (602-827-2265), Megan Gutierrez, Graduate Student (440-452-5142), or Bianca Teran, Graduate Student (520-370-2441).

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk; you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given (offered) to you.

Your signature below indicates that you consent to participate in the above study.

Subject's Signature	Printed Name	Date

Preferred contact: phone and/or email:

_____

INVESTIGATOR'S STATEMENT
"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX F

SUBJECT QUESTIONNAIRES

HEALTH /HISTORY QUESTIONNAIRE ID#_____

1. (To be completed by researchers): Height _____ Weight _____

   Percent body fat_____ BMI_____

2. Age: _____

3. Have you lost or gained more than 5 lbs in the last 12 months? Yes No

   If yes, how much lost or gained? _____ How long ago? _____

4. College Status (please circle) Fresh. Soph. Jr. Sr. Grad.

5. Ethnicity: (please circle) Native American African-American Caucasian Hispanic Asian Other

6. Do you smoke? No, never _____

   Yes _____ # Cigarettes per day = _____

   I used to, but I quit _____ months/years (circle) ago

7. Do you take any medications regularly? Yes No *If yes, list type and frequency:*

Medication	Dosage	Frequency

8. Do you currently take supplements (vitamins, minerals, herbs, etc.) ? Yes No *If yes, list type and frequency:*

Supplement	Dosage	Frequency

9. Have you ever been hospitalized? _____ If yes, for what? _____

OVER →

10. Please ANSWER (YES/NO) if **you currently have** or if **you have ever** been diagnosed with any of the following diseases or symptoms:

	YES	NO		YES	NO
Coronary Heart Disease			Chest Pain		
High Blood Pressure			Shortness of Breath		
Heart Murmur			Heart Palpitations		
Rheumatic Fever			Any Heart Problems		
Irregular Heart Beat			Coughing of Blood		
Varicose Veins			Feeling Faint or Dizzy		
Stroke			Lung Disease		
Diabetes			Liver Disease		
Low Blood Sugar			Kidney Disease		
Bronchial Asthma			Thyroid Disease		
Hay Fever			Anemia		
Leg or Ankle Swelling			Hormone Imbalances		
Eating Disorders			Emotional Problems		

Please elaborate on any condition listed above._____
_____

11. How would you rate your lifestyle?
Not active _____ Active _____
Somewhat active _____ Very Active _____

12. Please circle the total time you spend in each category for an average **week.**

Light activities such as:
Slow walking, golf, slow cycling, doubles tennis, easy swimming, gardening
Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Moderate activities such as:
Mod. Walking, mod. cycling, singles tennis, mod. swimming, mod. weight lifting
Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Vigorous activities such as:
Fast walking/jogging, fast cycling, court sports, fast swimming, heavy/intense weight lifting
Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

13. How much alcohol do you drink? (average drinks per day) _____

14. Do you have any food allergies? Yes No If yes, explain:_____

15. Do you follow a special diet? (weight gain/loss, vegetarian, low-fat, etc.) Yes No

If yes, explain: _____

Food Frequency Questionnaire

This form asks about your usual dietary intake over the past month. Read each food item. If you have not eaten this food in the past month, mark "none" and move onto the next food item. Indicate whether you think your usual serving size is small (S), medium (M), or large (L) by marking the correct serving size box. Think over the past month. How often do you usually eat each of the following food items? Answer each question as best you can; estimate if you are not sure. NOTE: A small (S) serving is equal to half (½) the usual serving. A medium (M) is equal to the medium servings listed on the form. A large (L) is equal to one and a half (1 ½) times as much or more of the medium serving.

Seafood & Fish	Medium Serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
Tuna	3 ounces											
Salmon	3 ounces											
Whitefish	3 ounces											
Herring	3 ounces											
Walleye	3 ounces											
Lake trout	3 ounces											
Rainbow trout	3 ounces											
Sablefish	3 ounces											
Mackerel	3 ounces											
Catfish	3 ounces											
Flounder	3 ounces											
Perch	3 ounces											
Atlantic cod	3 ounces											
Atlantic bluefish	3 ounces											
Atlantic sturgeon	3 ounces											
Halibut	3 ounces											
Swordfish	3 ounces											
Mussels	3 ounces											
Scallops	3 ounces											
Oysters	3 ounces											
Shrimp	3 ounces											
Sardines	3 ounces											
Anchovy	3 ounces											
Blue crab	3 ounces											
Northern lobster	3 ounces											

* 3 ounces is about the size of a deck of cards.

Meat	Medium serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
Turkey	3 ounces											
Chicken	3 ounces											
Beef	3 ounces											
Pork	3 ounces											

* 3 ounces is about the size of a deck of cards.

Eggs	Medium serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
Regular egg	1 egg											
Omega-3 enriched egg	1 egg											
Eggland's Best egg	1 egg											

Dairy products	Medium serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
2% milk	1 cup											
1% milk	1 cup											
Skim milk	1 cup											
Cheddar cheese	¼ cup											
Swiss Cheese	1 ounce											
Mozzarella Cheese	1 ounce											
2% fat cottage cheese	½ cup											
1% fat cottage cheese	½ cup											
Feta cheese	1 ounce											
Yogurt – no fat or low fat	8 ounces											

Nuts/seeds	Medium serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
Walnuts	1 ounce											
Pumpkin seeds	1 ounce											
Flaxseeds	1 ounce											
Butternuts	1 ounce											
Cashews	1 ounce											
Hickory nuts	1 ounce											
Beechnuts	1 ounce											
Almonds	1 ounce											
Pistachios	1 ounce											
Pine nuts	1 ounce											
Pecans	1 ounce											
Brazilnuts	1 ounce											
Sunflower seeds	1 ounce											
Sesame seeds	1 Tbsp											
Poppy seeds	1 Tbsp											

Fats and Oils	Medium serving	None	S	M	L	Once a month	Less than once a week	1-2 times a week	3-4 times a week	5-6 times a week	Daily	More than once a day
Miracle whip	1 tsp											
Margarine	1 tsp											
Soybean oil	1 tsp											
Sunflower oil	1 tsp											
Flax oil	1 tsp											
Canola oil	1 tsp											
Olive Oil	1 tsp											
Walnut oil	1 tsp											

Please check each day capsule is consumed:

January 2012

Sun	Mon	Tue	Wed	Thu	Fri	Sat
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

February 2012

Sun	Mon	Tue	Wed	Thu	Fri	Sat
			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29			

March 2012

Sun	Mon	Tue	Wed	Thu	Fri	Sat
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

INSTRUCTIONS

1. Check off each day indicating that the capsule was taken in the morning, preferably with food
2. Arrive to lab at scheduled times for testing; at weeks 4 and 8 bring booklet and remaining capsules

Contact the researchers if you have any questions at:

Dr. Carol Johnston: Carol.Johnston@asu.edu, 602-827-2265
Bianca Teran: bmteran@asu.edu, 520-370-2441
Megan Gutierrez: mgutie15@asu.edu, 440-452-5142

Wisconsin Upper Respiratory Symptom Survey – 21 — *Daily Symptom Report*

Day:	*Date:*	*Time* (To be completed at or near bedtime)	*ID:*

Please fill in one circle for each of the following items

	Not sick	Very mildly		Mildly		Moderately		Severely
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
In terms of respiratory tract illness only, how sick do you feel **today**?	O	O	O	O	O	O	O	O

Please rate the **average severity of your cold symptoms over the last 24 hours** for each symptom:

	Do not have this symptom	Very mild		Mild		Moderate		Severe
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Runny nose	O	O	O	O	O	O	O	O
Plugged nose	O	O	O	O	O	O	O	O
Sneezing	O	O	O	O	O	O	O	O
Sore throat	O	O	O	O	O	O	O	O
Scratchy throat	O	O	O	O	O	O	O	O
Cough	O	O	O	O	O	O	O	O
Hoarseness	O	O	O	O	O	O	O	O
Head congestion	O	O	O	O	O	O	O	O
Chest congestion	O	O	O	O	O	O	O	O
Feeling tired	O	O	O	O	O	O	O	O

Over the last 24 hours, how much has your cold interfered with your ability to:

	Not at all	Very mildly		Mildly		Moderately		Severely
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Think clearly	O	O	O	O	O	O	O	O
Sleep well	O	O	O	O	O	O	O	O
Breathe easily	O	O	O	O	O	O	O	O
Walk, climb stairs, exercise	O	O	O	O	O	O	O	O
Accomplish daily activities	O	O	O	O	O	O	O	O
Work outside the home	O	O	O	O	O	O	O	O
Work inside the home	O	O	O	O	O	O	O	O
Interact with others	O	O	O	O	O	O	O	O
Live your personal life	O	O	O	O	O	O	O	O

Compared to yesterday, I feel that my cold is: [If you did not have cold symptoms yesterday, please leave blank.]

Very much better	Somewhat better	A little better	The same	A little worse	Somewhat worse	Very much worse
O	O	O	O	O	O	O

Please list any products (including prescription or over-the-counter medicines, herbal preparations or supplements, and/or lozenges) taken to relieve respiratory symptoms.

Product name:	Dosage:	Time(s) taken:

Did you smoke any cigarettes today? **Yes** _____ **No** _____
Did you drink alcohol today? **Yes** _____ **No** _____

How many caffeinated beverages did you consume today? _____

Godin Leisure-Time Exercise Questionnaire

1. During the previous **7-Day period** (one week), how many times on the average did you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number).

	Times Per Week:
a) STRENUOUS EXERCISE **(HEART BEATS RAPIDLY)** (e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)	_____
b) MODERATE EXERCISE **(NOT EXHAUSTING)** (e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)	_____
c) MILD EXERCISE **(MINIMAL EFFORT)** (e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)	_____

2. During the previous **7-Day period** (one week), in your leisure time, how often did you engage in any regular activity **long enough to work up a sweat** (heart beats rapidly)?

OFTEN SOMETIMES NEVER/RARELY

1.☐ 2.☐ 3.☐

APPENDIX G

24-HOUR RECALL SCRIPT

24 Hour Dietary Recall Guide

Subject #_____

Upon waking, what food and beverages did you consume?

Food/Beverage	Quantity	Portion Size

What was the next thing you ate or drank?

Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for lunch?

Food/Beverage	Quantity	Portion Size

Did you have any snacks or beverages next?

Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for dinner?

Food/Beverage	Quantity	Portion Size

Did you eat or drink anything else throughout the day or night?

Food/Beverage	Quantity	Portion Size

Now I would like to go back and add more detail on how much of each food and beverage you consumed as well as how the food was prepared, starting with the first item.

Is there any condiment, topping, seasoning or food you may have missed, such as: sugar, butter, ketchup, salt, cream cheese, etc.?

Think for a minute. Was there any food, beverage or anything else you may have missed that you consumed yesterday?

All right, now I would like to go through the foods and beverages we have listed and make sure they are listed correctly.

Was this a typical day in terms of dietary choices and eating patterns? What differs?

APPENDIX H

ELISA PROCEDURE

Human TNF-a Immunoassay

REAGENT PREPARATION
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD6-35 (1X) (for cell culture supernate samples) - Add 20 mL of Calibrator Diluent RD6-35 to 80 mL of deionized or distilled water to yield 100 mL of Diluted Calibrator Diluent RD6-35.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 mL of the resultant mixture is required per well.

TNF-a Standard - Refer to vial label for reconstitution volume. Reconstitute the TNF-a Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 mL of Calibrator Diluent RD6-35 (for serum/plasma samples) or Calibrator Diluent RD6-35 (1X) (for cell culture supernate samples) into the 1000 pg/mL tube. Pipette 500 mL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 mL of Assay Diluent RD1F to each well. Assay Diluent RD1F will have a precipitate present. Mix well before and during use.
4. Add 200 mL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 mL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 mL of TNF-a Conjugate to each well. Cover with a new adhesive strip.
For Serum/Plasma Samples: Incubate for 2 hours at room temperature.
For Cell Culture Supernate Samples: Incubate for 1 hour at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 mL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**

9. Add 50 mL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

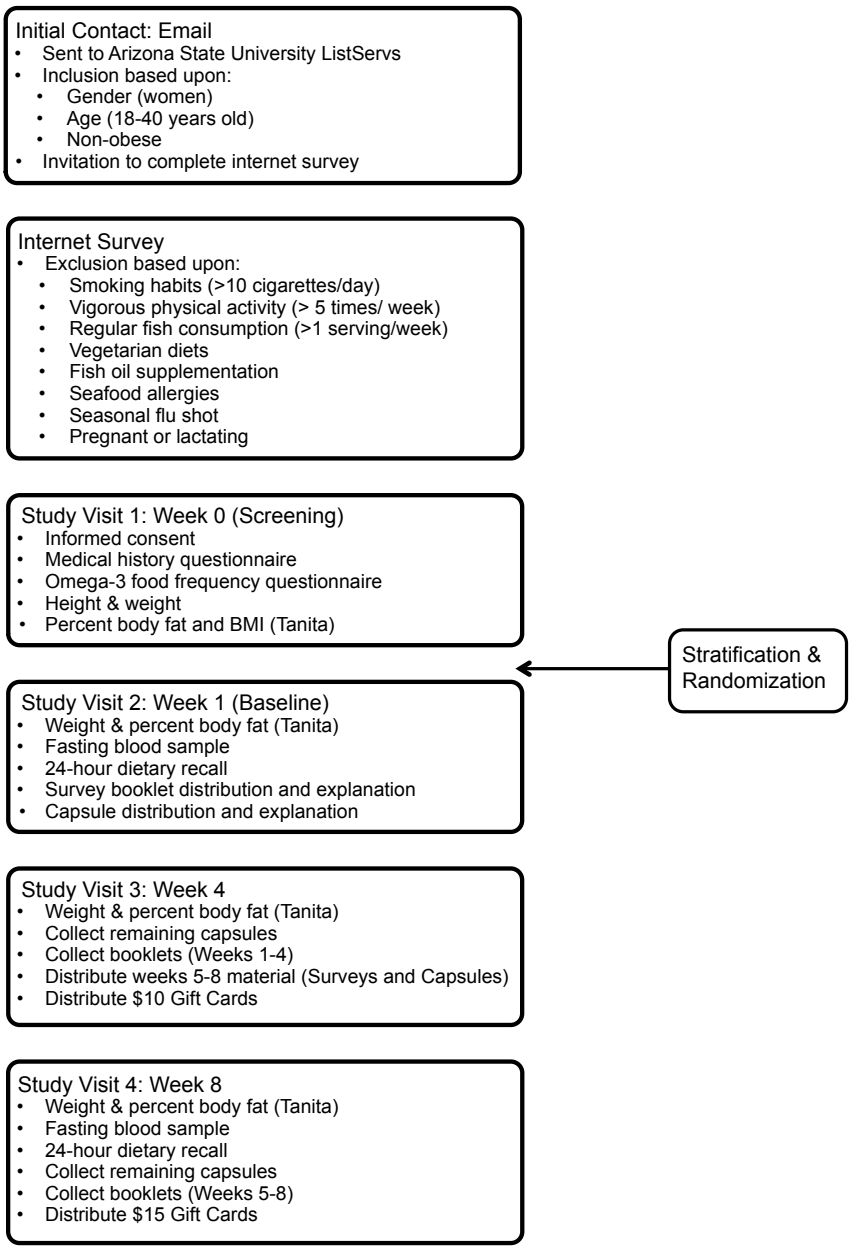
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TNF-a concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

APPENDIX I

METHODOLOGY TIMELINE


Initial Contact: Email
• Sent to Arizona State University ListServs
• Inclusion based upon:
• Gender (women)
• Age (18-40 years old)
• Non-obese
• Invitation to complete internet survey
Internet Survey
• Exclusion based upon:
• Smoking habits (>10 cigarettes/day)
• Vigorous physical activity (> 5 times/ week)
• Regular fish consumption (>1 serving/week)
• Vegetarian diets
• Fish oil supplementation
• Seafood allergies
• Seasonal flu shot
• Pregnant or lactating
Study Visit 1: Week 0 (Screening)
• Informed consent
• Medical history questionnaire
• Omega-3 food frequency questionnaire
• Height & weight
• Percent body fat and BMI (Tanita)
Stratification & Randomization
Study Visit 2: Week 1 (Baseline)
• Weight & percent body fat (Tanita)
• Fasting blood sample
• 24-hour dietary recall
• Survey booklet distribution and explanation
• Capsule distribution and explanation
Study Visit 3: Week 4
• Weight & percent body fat (Tanita)
• Collect remaining capsules
• Collect booklets (Weeks 1-4)
• Distribute weeks 5-8 material (Surveys and Capsules)
• Distribute $10 Gift Cards
Study Visit 4: Week 8
• Weight & percent body fat (Tanita)
• Fasting blood sample
• 24-hour dietary recall
• Collect remaining capsules
• Collect booklets (Weeks 5-8)
• Distribute $15 Gift Cards