The Effects of Long Term Walnut Consumption on Levels of Plasma Tocopherols and Inflammatory Mediators

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THE EFFECTS OF LONG TERM WALNUT CONSUMPTION ON LEVELS OF PLASMA TOCOPHEROLS AND INFLAMMATORY MEDIATORS

by
Suwimol Sapwarobol

A Dissertation in Partial Fulfillment of the Requirements for the Degree of Doctor of Public Health in Nutrition

June 2005
Each person whose signature appears below certifies that this dissertation, in his/her opinion, is adequate in scope and quality as a dissertation for the degree Doctor of Public Health.

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ABSTRACT OF THE DISSERTATION

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Loma Linda University, Loma Linda California, 2005

Ella H. Haddad, Chairman

The purpose of this study was to determine the effects of long term walnut consumption on plasma levels of tocopherols and the inflammatory mediators interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) in healthy participants. Ninety participants were recruited into a 12-month randomized crossover intervention study, which consisted of two phases, the walnut supplemented diet and the habitual diet. Participants were randomly assigned to either of these two phases for six months and then switched to the other phase for the second six months. During the walnut supplemented diet phase, participants incorporated walnuts (28-56 g/d) equal to 12% of their daily energy intake into their habitual diet. Plasma concentrations of α-tocopherols were significantly decreased but γ-tocopherol level was insignificantly increased during walnut supplemented diet. However, the ratio
of γ-tocopherol to cholesterol was significantly higher on the walnut supplemented diet. In addition, data from preliminary analysis on correlation of gender, age and BMI on CRP showed linear effect of BMI to log (CRP) (β = 0.088 ± 0.029, p = 0.002), however, no effect of gender and age on CRP levels were found. CRP levels were almost 50% lower in the group of NSAIDs and vitamin E supplement users. Moreover, the walnuts supplemented diet together with vitamin E supplement elevated CRP level by 22.72%. After excluding usage of fish oil, vitamin E supplement and NSAIDs, thirty participants were qualified for the inflammatory mediator analysis. There was no significant change in any of the plasma e-selectin and TNF-α level. However, IL-1β and IL-6 were significantly decreased during the walnuts supplemented diet (p < 0.05). In conclusion, the addition of walnuts for 12% of daily energy intake into the diet did not increase plasma γ-tocopherol, but decreased α-tocopherol level significantly. Moreover, IL-1β and IL-6 were decreased significantly with this amount of walnuts intake.


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CHAPTER 1

INTRODUCTION

A. Statement of the Problem

The importance of including walnuts (Juglans regia) in a healthful diet is widely acknowledged. According to prospective cohort studies such as the Adventist Health Study (Fraser et al., 1992), the Nurses Health Study (Hu et al., 1998), the Iowa Woman’s Health Study (Kushi et al., 1996) and the Physician’s Health Study (Morgan et al., 2002), more frequent nut consumption is correlated with cardiovascular risk reduction and is inversely associated with all cause mortality. Clinical human intervention studies which involve adding walnuts to the diet are consistent in showing decreases in total cholesterol and Low Density Lipoprotein (LDL)-cholesterol (Sabaté et al., 1993; Abbey et al., 1994; Chilsholm et al., 1998; Zambón et al., 2000; Almario et al, 2001). The favorable metabolic outcomes of consuming walnuts is thought to be due to a lipid composition low in saturated fatty acids (SFA) and high in polyunsaturated fatty acids (PUFA). Although the nutrient contribution of walnuts is similar to that of other tree nuts, it is distinguished by a high proportion of the n-3 fatty acids, α-linolenic acid and of γ-tocopherol.

Beneficial health effects of n-3 fatty acids have received considerable interest ever since it was reported that coronary atherosclerosis is almost unknown among Greenland Eskimos living in their original cultural environment and consuming primarily a fish diet rich in n-3 fatty acids as compared to those living in Denmark. The n-3 fatty acids have a wide range of physiological effects. Those relevant to heart disease are influences on blood lipids, endothelial function and cytokine production.
Studies have shown that long term supplementation of capsules from fish oil containing the long chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduced production of proinflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) in healthy volunteers (Endres et al., 1989; Calder 1997; Kew et al., 2003; Venkatraman et al., 1999). The inhibition of IL-1, IL-6 and TNF production is accompanied by a decreased ratio of arachidonic acid (AA) to EPA in the membrane phospholipids of mononuclear cells (Endres et al., 1989). EPA can be formed endogenously from the 18-carbon fatty acid α-linolenic acid (18:3,n-3). Flaxseed oil, which contains a high proportion of α-linolenic acid, inhibited TNF-α and IL-1β production to a lesser extent ~30% compared to a >70% inhibition achieved using concentrated fish oil capsules (Caughey et al., 1996). The effect of walnut lipids on proinflammatory mediator synthesis has not been examined.

In addition to n-3 PUFA, walnuts are a rich source of γ-tocopherol, which may also have a role in inflammatory response. The tocopherols including γ-tocopherol are antioxidant scavenging compounds known to inhibit phospholipid oxidation and the oxidation of LDL components. Conditions associated with increased intracellular oxidative stress facilitate nuclear translocation of the transcriptional factor Nuclear Factor (NF)-κB and induce the production of proinflammatory cytokines (Flohe et al., 1997; Asehnoune et al., 2004). Studies have shown that α-tocopherol supplementation inhibits NF-κB activation and inflammation in vivo (Devaraj et al., 1996; Blackwell et al., 1996). More recently Li et al (1999) showed that treatment of human coronary artery endothelial cells with γ-tocopherol attenuated the oxidized-LDL mediated activation of NF-κB. It is
not known whether the addition of walnuts to the diet would increase blood levels of \( \gamma \)-tocopherol and inhibit the inflammatory response by protecting cells from oxidative challenge.

Besides the inflammatory cytokines, C-reactive protein (CRP) has also been investigated in coronary vascular disease (CVD). As an acute phase reactant, CRP is a non-specific marker of inflammation. Evidence suggested that CRP may have a direct proinflammatory effect involved in CVD (Wadham et al., 2004). CRP was consistently shown to be a strong predictor of CVD (Ridker et al., 1998; Ridker et al., 2002).

Research suggests that protective effect of nuts in the diet is due to their lipid-lowering potential. However, nuts also contain substantial amounts of tocopherols and some nuts, especially walnuts, are rich sources of \( \alpha \)-linolenic acid. According to Continuing Survey of Food Intakes by Individuals, CDFII (1994-96) current US dietary intake of linoleic acids (18:2, n-6) to \( \alpha \)-linolenic acid (18:3, n-3) is 9.5:1, whereas a current recommendation is a ratio of 5:1. Marine sources of n-3 fatty acids are problematic to some due to concentrations of environmental contaminants. The long-term safety of flaxseed and flaxseed oil consumption has not been established. Walnuts, however, have been an important ingredient in cultural diets of Mediterranean countries for centuries. There is as yet no information regarding the association between walnuts ingestion, antioxidant status and proinflammatory cytokines production. It remains to be determined whether the addition of walnuts to the diet produces similar effects on reduction of proinflammatory mediators and cytokines as those observed in fish and flaxseed oil via enhancing antioxidant status.
B. Purpose of the Study

The purpose of this study is to further explore the role of nuts in the diet beyond that of blood lipids. More specifically, it is to determine whether the addition of walnuts to the habitual diet influences markers of antioxidant and the inflammatory response in healthy free-living adults. Reduced oxidative stress and inflammation may explain some of the positive health outcomes associated with walnuts consumption.

C. Objective

Specifically, the objective of this study is to examine the effect of incorporating walnuts into diets on plasma markers of: (1) antioxidant status assessed by measuring levels of α-, β-, γ-, and δ- tocopherol; and (2) inflammation assessed by measuring levels of the proinflammatory cytokines IL-1β, IL-6, TNF-α and CRP. The study will also seek to determine the correlation between tocopherols and inflammatory mediators.

D. Research Questions

The study will focus on the following research questions:

(1) How does daily walnut consumption influence the levels of plasma tocopherols?

(2) How does daily walnut consumption influence proinflammatory cytokines and CRP production?

(3) Is there any correlation between levels of plasma tocopherols after walnuts consumption and proinflammatory cytokines in healthy people?
CHAPTER 2
REVIEW OF THE LITERATURE

A. Introduction

The concept that natural foods, or their components, offer benefits beyond basic nutrition is relatively new to the scientific community. This knowledge continues to be supported by research, which demonstrates that a health promoting diet is defined as much by foods consumed as by those avoided.

Epidemiologic evidence for the protective action of nut consumption in heart disease prevention was first observed by Fraser, Sabatè and colleagues in the Adventist Health Study cohort. This association was strengthened as evidence emerged from various observational and intervention studies on nuts.

Walnuts (Juglans regia L.) are traditional nuts in diets of the Mediterranean region, South America and Asia. They are one of the ancient tree foods known to humans. Although nuts are included in the USDA’s Food Guide Pyramid as part of the “Meat, Dry Beans, Eggs and Nuts” group, the per capita consumption of nuts is relatively low in the United States (US) as compared to other countries. Due to their caloric density and high fat content, walnuts were not commonly considered to be an integral component of a healthy diet, however, this concept is changing.

Walnuts contain numerous beneficial nutritive and bioactive compounds including polyunsaturated fatty acids, dietary fiber, trace minerals and phytochemicals, many of which may be cardioprotective (Table 1). Walnuts are a rich source of tocopherols, especially γ-tocopherol, which may be an important anti-atherogenic agent. (Spiller et al., 1998; Ros et al., 2004; Zambón et al., 2000; Almario 2001)
Table 1. Nutrient Composition of Walnuts

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Units</th>
<th>Value per 100 g of edible portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>Kcal</td>
<td>654</td>
</tr>
<tr>
<td>Protein</td>
<td>G</td>
<td>15.23</td>
</tr>
<tr>
<td>Total lipid (fat)</td>
<td>G</td>
<td>65.21</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>G</td>
<td>6.7</td>
</tr>
<tr>
<td>Calcium, Ca</td>
<td>Mg</td>
<td>98</td>
</tr>
<tr>
<td>Iron, Fe</td>
<td>Mg</td>
<td>2.91</td>
</tr>
<tr>
<td>Magnesium, Mg</td>
<td>Mg</td>
<td>158</td>
</tr>
<tr>
<td>Phosphorus, P</td>
<td>Mg</td>
<td>346</td>
</tr>
<tr>
<td>Potassium, K</td>
<td>Mg</td>
<td>441</td>
</tr>
<tr>
<td>Sodium, Na</td>
<td>Mg</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>IU</td>
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</tr>
<tr>
<td>Thiamin</td>
<td>Mg</td>
<td>0.341</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>Mg</td>
<td>0.150</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Mg</td>
<td>1.3</td>
</tr>
<tr>
<td>Niacin</td>
<td>Mg</td>
<td>1.125</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>Mg</td>
<td>0.570</td>
</tr>
<tr>
<td>Folate</td>
<td>Mcg</td>
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<td>Alpha-tocopherol</td>
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<td>0.70</td>
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<td>Beta-tocopherol</td>
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<td>Gamma-tocopherol</td>
<td>Mg</td>
<td>20.83</td>
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<tr>
<td>Delta-tocopherol</td>
<td>Mg</td>
<td>1.89</td>
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<tr>
<td>Fatty acids, total saturated</td>
<td>G</td>
<td>6.126</td>
</tr>
<tr>
<td>16:0</td>
<td>G</td>
<td>4.404</td>
</tr>
<tr>
<td>18:0</td>
<td>G</td>
<td>1.659</td>
</tr>
<tr>
<td>20:0</td>
<td>G</td>
<td>0.063</td>
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<tr>
<td>Fatty acids, total monounsaturated</td>
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<td>8.933</td>
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<td>18:1 undifferentiated</td>
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<td>8.799</td>
</tr>
<tr>
<td>20:1</td>
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<tr>
<td>Fatty acids, total polyunsaturated</td>
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<td>38.093</td>
</tr>
<tr>
<td>18:3 undifferentiated</td>
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<td>9.080</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>Mg</td>
<td>72</td>
</tr>
</tbody>
</table>

US. Department of Agriculture
As shown above, walnuts are a rich source of both α-linolenic acid (ALA), an n-3 fatty acid (9.1 g/100 g edible portion) and linoleic acid (LA), an n-6 fatty acid (38.1 g/100g edible portion). According to the above table, the ratio of linoleic to α-linolenic acid (4.2:1) in walnuts is the ratio that has been recommended.

The Continuing Survey of Food Intakes by Individuals 1994-1996 (CSFII 1994-1996) stated that current US dietary intake of linoleic acid to α-linolenic acid is 9.5:1 whereas the recommended ratio is 5:1. Thus, the addition of walnuts into the diet is the simple and uncomplicated way expected to move the ratio of linoleic acid to α-linolenic acid closer to the ideal.

The purpose of this literature review is two-fold. First, it is to summarize outcomes reported in published studies on the effects of long term incorporation of walnuts into the habitual diet. Second, it is to discuss other potential health benefits of walnuts which are expected based on their unique nutrient contents and non-nutrient composition.

B. Studies on Walnut Consumption

1. Observational Studies

Up to date, dietary alteration is believed to be a highly cost-effective method for cardiovascular disease (CVD) prevention. A number of studies have identified several dietary determinants of coronary heart disease (CHD), including specific type of fat, folate, vitamins, dietary fiber and moderate alcohol consumption. Recently, a number of observational studies on the association of nut consumption and CHD risk reduction have been published. They consistently suggest that frequent nut consumption may be protective against CHD.
Nuts are complex plant foods that are not only rich sources of unsaturated fat but also contain several non-fat constituents such as plant protein, fiber, micronutrients, plant sterols and phytochemicals. However, the consumption of nuts in the United State has been declining since the mid-1980s, because of the concern with their high-fat content.

Four prospective cohort studies, namely the Adventist Health Study, the Iowa Woman Health Study, the Nurses Health Study, and the Physician's Health Study, have consistently shown the health benefits of nuts consumption on the prevention of heart disease.

The protective effect of nuts on CHD morbidity and mortality was first suggested by data from the Adventist Health Study, a large prospective cohort investigation of 31,208 non-Hispanic white Seventh-day Adventists in California. (Fraser et al., 1992). After 6 years follow up, subjects were evaluated for first event myocardial infarction (MI), or CHD. The finding suggested that people who consumed nuts 1-4 times per week had a 22% reduced risk of MI compared to those that consumed nuts less than once a week. Moreover, people who consumed nuts four times a week or more experienced fewer fatal CHD events (relative risk 0.52; 95% confidence interval, 0.36 to 0.76) and nonfatal MI (relative risk, 0.49; 95% confidence interval, 0.28 to 0.85) when compared to those who consumed nuts less than one time a week.

Subsequent analysis of data from the Adventist Health Study (Fraser et al., 1995; Fraser 1999; Sabate, 1999) showed that the protective effect of nut consumption on CHD was consistent in both men and women, in younger and older individuals, and in people with normal and high blood pressure. Furthermore, the same protective effect on CHD was demonstrated in people regardless of their relative weight, smoking status, or
physical activity level. When assessing lifetime risk of developing CHD and age at first coronary event, Fraser et al. (1995) estimated that people who consumed nuts more than 5 times per week had a 12% lower lifetime risk of heart disease. Additionally, among men who experienced CHD those that consumed walnuts had the event 5.6 years later than did men who consumed nuts infrequently.

The Iowa Women’s Health Study is a prospective cohort study that followed 34,486 postmenopausal women for a total of 7 years to determine the effect of long term nut consumption on CHD mortality (Kushi et al., 1996). Over the 5 years follow up, 154 women who had been free of CHD at the baseline died of CHD. The CHD mortality was inversely associated with nuts intake. Over the total of 7 years follow up, 242 women died of CHD. In this study, multivariate analysis of the association between risk of death from CHD and intake of specific foods in women who did not take supplements showed a relative risk of 0.60 (95% confidence interval 0.35-1.01) in those who consumed nuts and seeds more than 4 times a month compared to those who never consumed nuts.

Another epidemiologic support of association between nut consumption and heart disease comes from the Nurses Health Study (Hu. et al., 1998; Hu et al., 1999) which focused on the association between nut consumption and nonfatal MI. Participants of this study were 86,016 female registered nurses aged 34 – 59 y. residing in 11 large US states. This study had shown that nurses who consumed at least 5 oz. of nuts per week had a 35% lowering in nonfatal MI compared to those eating less than 1 oz. of nuts per month. In this study 1255 cases of major coronary disease events were documented during 14 years of follow-up. Results after adjusting for age, smoking, and other known risk factors for coronary heart disease demonstrate that women who ate more than 5 oz (140 g) of
nuts a week had a significantly lower risk of CHD (relative risk 0.65, 95% confidence interval 0.47 to 0.89) than women who never ate nuts or who ate less than 1 oz (28 g) a month.

In the Physician's Health Study (Albert et al., 1998) 21,454 male physician participants were followed prospectively for 17 years to examine the association between nut consumption and risk of sudden death and total cardiac death. After controlling for known cardiac risk factors, dietary nut intake was associated with a significantly reduced risk of sudden cardiac death. Compared with men who rarely or never consumed nuts, those who consumed nuts 2 or more times per week had reduced risk of sudden cardiac death (RR= 0.53; 95% CI= 0.30-0.92) and total CHD mortality (RR= 0.70; 95% CI = 0.50-0.98). In this study nut intake was not associated with reduced risk of nonsudden CHD or nonfatal MI. The investigators suggested that as nut consumption increased the risk for cardiac and sudden cardiac deaths decreased significantly.

In summary, four large prospective cohort studies have agreed that the frequency of nut consumption seems to be inversely related to the risk of CHD. These beneficial effects are similar in males and females, and the young and the elderly.

2. Clinical Intervention Studies

The epidemiologic evidence indicating an inverse association between consumption of nuts and risk of CHD provided the impetus for intervention trials designed to investigate the mechanisms underlying this effect. These studies assessed the effect of feeding a single type of nut (pecan, almond, walnut, peanut) either as a supplement in a field trial or under carefully controlled metabolic conditions.
The effects of walnut consumption on biomarkers of atherosclerosis were evaluated in five controlled studies in primarily healthy and in normal or hyperlipidemic subjects. These studies are presented here in chronological order of publication.

The first of the nuts to be tested in a controlled feeding study in human was walnuts. Sabaté et al. (1993) studied the effects of walnuts consumption on serum lipids and blood pressure in 18 free-living healthy, normal weight and normolipidemic adult males. The participants were randomly placed in a National Cholesterol Education Program (NCEP)/American Heart Association (AHA) step1 diet, either with or without walnuts supplementation at 20% of daily calories. The participants were requested to follow both diets for four weeks each in a randomized crossover design.

Results have shown that during the walnut supplemented diet, total cholesterol, low density lipoprotein cholesterol (LDL) and high density lipoprotein cholesterol (HDL) levels were significantly decreased by 12%, 16% and 5% respectively; all changes were statistically significant. The investigators concluded that replacing a portion of the fat in a cholesterol-lowering diet with walnuts produces more favorable lipoprotein profile and further reduces serum cholesterol level and that the average consumption of walnuts 28 g per day would lower total-C and LDL-C by 4 and 6% respectively.

Abbey et al. (1994) also examined the effects of nut consumption on blood lipids profile. This study was conducted on 16 normolipidemic males with a 36% fat diet for 3 weeks period. In this study, the investigators intended to compare three diets: one enriched with peanuts and coconuts which provided 47 g of both monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA); an almond enriched diet (84 g of almond per day) which provided mainly MUFA; and, a walnut enriched diet (68 g of
walnuts per day) which provided mainly PUFA. Sixteen normalipidemic males were assigned to follow these three diet regimens for three weeks each. The dietary intake was monitored and verified by food records, with special emphasis on different type of fat intake.

After 3 weeks of each diet, the results indicated that both almond and walnut enriched diets significantly reduced total-C and LDL-C by 6 and 10% respectively. There was no significant difference in HDL-C and triacylglycerol values among those three diets. The plasma linoleic and α-linolenic acids were significantly increased. The investigators suggested that PUFA and MUFA rich nuts should be included in the diets as a replacement for some of the SFA for favorable health effects.

Chisholm et al. (1998) also studied the effects of walnuts consumption (78 g/day) on lipid metabolism in 21 males with moderate hypercholesterolemia. Walnuts were included into the 38% “LF” diet. After 4 weeks follow up, total-C and LDL-C were decreased by 4 and 8% respectively in the walnut diet compared to baseline. In contrast, HDL-C was increased significantly by 42% with the walnuts supplemented diet. Triacylglycerol linolenate also increased significantly. In this study, fatty acid profile of the major lipid fractions after walnuts ingestion showed changes which might be expected to reduce risk of cardiovascular disease.

In 2000, Zambón et al., conducted a randomized, crossover feeding study to determine the effect of different types of fat on blood lipid profiles. This study was carried out on 49 men and women participants with polygenic hypercholesterolemia. This study was also designed to assess the acceptability of walnuts and their effects on serum lipid levels and LDL-oxidizability in free-living hypercholesterolemia patients. In a high
PUFA diet, walnuts contributed approximately 18% of the total energy intake and 35% of the total fat. A high MUFA Mediterranean diet served as control. After 6 months consumption of each diet, examinations of blood lipid profiles were performed. Walnut diet produced mean changes of -4.1% in total cholesterol level, -5.9% in LDL, and -6.2% in lipoprotein(a) level compared to the control diet. In addition, reductions of total cholesterol and LDL-C by 9 and 11% respectively were observed in the walnut diet. There was, however, no significant difference in HDL-C or triacylglycerol in the walnuts supplemented diet compared to control diet. Moreover, during the walnuts supplemented diet the ability of LDL resistance to oxidative stress was preserved. Investigators concluded that incorporating walnuts for part of a high MUFA cholesterol-lowering diet further reduces total cholesterol and LDL-C in subjects with hypercholesterolemia.

In 2001, Almario et al conducted a study to determine the effects of walnut intake on plasma fatty acids and lipoproteins in patients with combined hyperlipidemia. In this study, four types of diet were examined: the habitual diet (31% fat) and a low fat diet (20% fat) and these two diets supplemented with walnuts (48 g). Thirteen males and females completed the four diets. The results showed that during the walnut supplemented low fat diet, plasma total cholesterol concentrations decreased by $0.58 \pm 0.16$ mmol/L when compared with the habitual diet and by $0.46 \pm 0.14$ mmol/L when compared with the low fat diet. In addition, the data indicated that incorporating walnuts (48 g) to a low fat diet decreased total cholesterol and LDL-C by 8 and 12% respectively when compared with those on the low fat diet alone. HDL-C level, however, decreased significantly by 10% with this amount of walnuts supplementation. The investigators,
therefore, noted that the cholesterol-lowering effect caused by adding walnuts to the low fat diet could be explained by the specific effect of the PUFA and MUFA content.

The findings of these five clinical human intervention studies consistently demonstrated decrease in total cholesterol and LDL-C levels with consumption of walnuts two to three servings daily. The main n-3 PUFA in walnuts, α-linolenic acid (ALA; 18:3n-3), is believed to be the cause of these beneficial health effects of walnuts. ALA is the essential precursor of n-3 PUFAs [eicosapentaenoic acid (EPA; 20:5 n-3), and docosahexaenoic acid (DHA; 22:6n-3)] found mostly in fish oils. It could also be elongated and desaturated in the human body to form long chain PUFA. Therefore, the effects of walnut on lowering both total cholesterol and LDL-C level as proved in fish oils are acknowledged. These five studies also proved the potential health benefit of walnuts in CHD risk reduction. Moreover, these effects have been accomplished whether walnuts were incorporated in a habitual diet, cholesterol lowering diet step 1, a low fat diet or a diet already high in PUFA. The effect of walnuts consumption on increasing HDL-C level, however, was inconsistent in these intervention studies.

Even though there are many proposed biological mechanisms of walnuts intake on blood lipid profile, the clear biological mechanism remains unclear. Since walnuts are a rich source of PUFA, the high bioavailability of PUFA in the body after walnuts consumption is one of the major suggested biological mechanisms. A possible effect of walnuts on homocysteine also needs to be examined. Similarly, the effect of arginine contained in walnuts may also be significant but remains unclear.
C. Potential Health Benefits of Walnut Components

1. Gamma-Tocopherol

Walnuts contain a high concentration of \( \gamma \)-tocopherol, which is one of a group of substances identified more than eighty years ago as factors essential for reproduction in female rats. Reproductive failure was observed in female rats fed semi-purified diets that otherwise contained adequate amounts of vitamins A, B-complex, C and D, and those other vitamin and minerals that supported growth and general health, indicating lack of an essential nutrient.

The word tocopherol came from Greek “tokos” mean childbirth, “phorein” mean to bring forth, and “ol” for the alcohol portion of the molecule. To represent this group of factors, the term vitamin E was officially accepted in 1925, as the fifth serial alphabetical designation for vitamins. Vitamin E was formally recognized as an essential nutrient for humans by inclusion in the Recommended Dietary Allowances (RDA) table of the Food and Nutrition Broad, National Academy of Sciences in 1968 (Kasperek 1980).

Originally, the term “vitamin E” was the generic name applied to the family of 8 different but structurally related tocopherol and tocotrienol derivatives (chromanols) that are produced exclusively by plants and that qualitatively exhibit the biologic activity of \( \alpha \)-tocopherol. Four belong to the tocopherol family: \( \alpha \)-, \( \beta \)-, \( \gamma \)- and \( \delta \)-tocopherols; the other four are tocotrienols: \( \alpha \)-, \( \beta \)-, \( \gamma \)- and \( \delta \)-tocotrienol. Structurally, the tocopherols and tocotrienols differ at their phytol side chain that tocopherols have a saturated phytol side chain with 3 chiral centers that are in an RRR configuration at positions 2, 4', and 8' in the naturally occurring forms; whereas, tocotrienols have an unsaturated side chain. The
eight forms of tocopherols are not interconvertible in mammalian and human systems (Kasparek 1980).
Figure 1. Structures of the naturally occurring forms of the tocopherols and tocotrienols
(Kasparek 1980)
The Food and Nutrition Board at the National Academy of Sciences recently released updated Dietary Reference Intakes (DRIs) for vitamin E. The committee recommended DRIs based exclusively on the $\alpha$-tocopherol form of vitamin E, because it is the only form recognized by the $\alpha$-tocopherol transport protein in the liver, preferentially secreted into the plasma, and maintained by the body. (Dietary Reference Intakes, 2000)

**a. Gamma-Tocopherol in the Diet and in Walnuts.** Even though the vitamin E requirement is based exclusively on $\alpha$-tocopherol, the most prevalent and abundant form of vitamin E in plants is $\gamma$-tocopherol. As a result, approximately 70% of the tocopherols consumed in the typical US diet is $\gamma$-tocopherol. Most of the $\gamma$-tocopherol in the diet comes from vegetable oils such as corn, soybean, sesame seed, and peanut oils and their derivative products such as margarine and salad dressings. In contrast, $\alpha$-tocopherol is contained mainly in almond, wheat germ and sunflower oil (McLaughlin 1979).
Table 2. Tocopherols Content in Foods

<table>
<thead>
<tr>
<th>Dietary</th>
<th>α</th>
<th>B</th>
<th>γ</th>
<th>δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bread</td>
<td>0.04</td>
<td>0.02</td>
<td>0.024</td>
<td>0.1</td>
</tr>
<tr>
<td>White</td>
<td>0.16</td>
<td>0.15</td>
<td>0.38</td>
<td>0.2</td>
</tr>
<tr>
<td>Whole wheat</td>
<td>0.60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fish</td>
<td>11.70</td>
<td>-</td>
<td>29.00</td>
<td>8.1</td>
</tr>
<tr>
<td>Margarine</td>
<td>9.7</td>
<td>-</td>
<td>6.60</td>
<td>-</td>
</tr>
<tr>
<td>Seeds and Nuts</td>
<td>25.87</td>
<td>0.43</td>
<td>0.89</td>
<td>0.25</td>
</tr>
<tr>
<td>Peanuts</td>
<td>34.50</td>
<td>1.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Almonds</td>
<td>0.70</td>
<td>0.15</td>
<td>20.83</td>
<td>1.89</td>
</tr>
<tr>
<td>Sunflower seeds</td>
<td>0.09</td>
<td>-</td>
<td>0.20</td>
<td>-</td>
</tr>
<tr>
<td>Walnuts</td>
<td>13.43</td>
<td>5.00</td>
<td>60.20</td>
<td>1.80</td>
</tr>
<tr>
<td>Oils</td>
<td>38.7</td>
<td>-</td>
<td>17.40</td>
<td>24.00</td>
</tr>
<tr>
<td>Safflower</td>
<td>133.00</td>
<td>71.00</td>
<td>26.00</td>
<td>27.1</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>38.90</td>
<td>-</td>
<td>38.70</td>
<td>-</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>14.35</td>
<td>0.11</td>
<td>0.83</td>
<td>-</td>
</tr>
<tr>
<td>Olive</td>
<td>0.09</td>
<td>-</td>
<td>0.20</td>
<td>-</td>
</tr>
</tbody>
</table>

US. Department of Agriculture

The tocopherols content of diets may shows great variation depending upon harvesting, processing, storage and final food preparation procedures.

b. Absorption, Transport and Distribution. The absorption of tocopherols depends upon factors that are commonly important in lipid digestion and intestinal uptake. In addition, the degree of absorption varies with total lipid absorption. The efficacy of the absorption; however, decreases as large amounts of tocopherols are consumed. In 1972, Losowsky et al. demonstrated that the absorption rate of tocopherols declined from 60% when 0.04 mg of α-tocopherol was administered to 30% when 20 mg were given. In healthy individuals, the absorption rate of 50-70% can be reached for
dietary levels of α-tocopherol ranging from 0.4 mg to 1 mg. However, the efficacy falls to less than 10% when pharmacologic doses of 200 mg or more are administered.

The utilization of deuterium-labeled tocopherols has facilitated the understanding of the absorption and transport of tocopherols (Kayden et al., 1993). Summarization of current knowledge of the absorption and metabolism of α- and γ- tocopherol are shown below.
Figure 2. The absorption and transport pathway of α-, and γ- tocopherol (Jiang et al., 2001)
All forms of tocopherols are taken up without any preference by intestinal cells after digestion and released into the circulation with chylomicrons. In general, the quantity absorbed varies depending on the amount of the lipid present (Kayden et al., 1993; Traber et al., 1992; Traber et al., 1989). During chylomicron transport, some of the tocopherol forms are presumably released to peripheral tissues including brain, lung, heart, skeleton muscle and adipose tissues with the aid of lipoprotein lipase.

The tocopherols appear to reach the liver by chylomicron remnants. In the liver, $\alpha$-tocopherol is preferentially reincorporated into nascent very low density lipoprotein (VLDL) by a mechanisms linked to $\alpha$-tocopherol transport protein ($\alpha$-TTP). Other tocopherol forms are much less well retained and are oxidized to carboxyethyl hydroxychromans (CEHC), and excreted via the bile, the urine, or other unknown routes.

Studies have demonstrated a constant fractional rate of $\alpha$-tocopherol absorption in healthy individuals. This rate determines a normal $\alpha$-tocopherol concentration in plasma of approximately 25 $\mu$mol per liter. Plasma tocopherol concentrations could be increased only by 2-3 fold when subjects are fed high levels of $\alpha$-tocopherol (Dimitrov et al., 1991; Jialal et al., 1995). Studies have determined the underlying explanations for this observation including variations in $\alpha$-TTP activity, metabolic rate, lipid content and lipid composition (Traber et al., 1998; Stahl et al., 1999; Roxborough et al., 2000).

In general, tocopherols are apparently concentrated wherever fatty acids abundant, especially in structures of the cell containing phospholipid membranes, such as mitochondria, and microsomes. The major total body pool of vitamin E is in adipose tissue; however, it is not readily available to other tissues. The liver, instead, is a major
repository when the intakes of tocopherols are high; therefore, the amount present in the liver is considered an index of dietary intake (Jiang et al., 2001).
Table 3. Comparison of Concentration of α- and γ-Tocopherols in the Human Plasma and Other Tissues

<table>
<thead>
<tr>
<th></th>
<th>γ- tocopherol</th>
<th>α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (µmol/L)</td>
<td>2-7</td>
<td>15-20</td>
</tr>
<tr>
<td>Adipose (nmol/g)</td>
<td>176 ± 80</td>
<td>440 ± 279</td>
</tr>
<tr>
<td>Muscle (nmol/g)</td>
<td>107</td>
<td>155 ± 163</td>
</tr>
<tr>
<td>Skin (nmol/g)</td>
<td>180 ± 89</td>
<td>127 ± 74</td>
</tr>
</tbody>
</table>

(Jiang et al., 2001)
c. Degradation. Tocopherols are extensively metabolized before excretion. Parker et al., 2000 reported that the degradation of tocopherols is by a cytochrome P450 3-A (CYP3A) dependent process. This was proven by the inhibition of \(\gamma\)-CEHC formation by sesamin and ketoconazole, inhibitors of the CYP3A family. Therefore, clinical studies have suggested that drugs that interfere with metabolism of CYP3A system should be considered during vitamin E supplementation. Although the involvement of CYP3A in the degradation process of vitamin E is clear, a non CYP3A is also responsible for tocopherol metabolism. The side effect of supranutritional dosages of vitamin E may inhibit platelet aggregation. Thus it has been recommended that anticoagulants and vitamin E supplements should not be provided simultaneously (Li et al., 2001).

d. Antioxidant Properties. Most of the recent interest in the tocopherols has focused on their antioxidant properties. The antioxidant activity of the tocopherols and tocotrienols is believed mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. As a consequence, tocopherols are widely promoted to prevent or modulate diseases that are supposedly associated with oxidative stress such as cardiovascular disease, cancer, chronic inflammation and neurological disorders (Christen et al., 1999; Ames et al., 1993; Stephens et al., 1996).

The various tocopherols and tocotrienols inhibit lipid peroxidation in both foods and biological systems. In biological systems, they function as chain breaking antioxidants preventing propagation of free radical damage in plasma and in membranes (Farrel et al., 1994; Rock et al., 1996). Chromanols react with both peroxyl and hydroxyl radicals and are effective agents to prevent both internal and external oxidative stress.
The chain-breaking antioxidant functions of vitamin E components results from their close association with the polyunsaturated fatty acids (PUFA) found in the membranes.

Vitamin E is a potent peroxyl radical scavenger. When lipid hydroperoxides are oxidized to peroxyl radicals (ROO'), they react 1000 times faster with vitamin E than with polyunsaturated fatty acids (RH). The hydroxyl group of tocopherol reacts with an organic peroxyl radical, forming hydroperoxide (ROOH) and the tocopherol radical (Vit E-O') (Traber et al., 1999).

With vitamin E

\[ \text{ROO'} + \text{Vit E-OH} \rightarrow \text{ROOH} + \text{Vit E-O'} \]

Without vitamin E

\[ \text{ROO'} + \text{RH} \rightarrow \text{ROOH} + \text{R'} \]
\[ \text{R'} + \text{O}_2 \rightarrow \text{ROO'} \]

Vitamin E, therefore, acts as a chain breaking antioxidant during propagation phase, preventing the further autooxidation of lipids. The tocopheroxyl radical (Vit E-O') then reacts with vitamin C (hydrogen donors, AH) thereby oxidizing and returning vitamin E to its reduced states.

\[ \text{Vit E-O'} + \text{AH} \rightarrow \text{Vit E-OH} + \text{A'} \]

The reducing agents, vitamin C, continuously repair oxidized tocopherol preventing loss of it. The tocopheroxyl radical could also be reduced by glutathione, or by membrane-bound quinols as well.
Although all of the tocopherols are effective lipophilic antioxidants, \( \gamma \)-tocopherol provides a unique function. Because of an unsubstituted 5-position on the chromanol ring, \( \gamma \)-tocopherol could effectively remove reactive nitrogen species. (Christen, 1997)

The main membrane antioxidant tocopherols consists of two major forms, \( \alpha \)-tocopherol and \( \gamma \)-tocopherol, that differ structurally only by a methyl group substitution at the 5-position. Gamma-tocopherol is the principal form of vitamin E in the diet, whereas \( \alpha \)-tocopherol is the more potent antioxidant and is the primary form in supplements.

Study of general antioxidant properties of \( \gamma \)-tocopherol suggest that accumulation of \( \gamma \)-tocopherol may be protective against both general oxidation reactions and nitrogen dioxide (NO\(_2\))-mediated damage. Animals are exposed to nitrogen oxides from a variety of sources. Endogenous nitric oxide (NO) is produced by macrophages in response to infection, by endothelial cells to control vascular tone, whereas a major exogenous exposure to NO is cigarette smoke. Nitrogen dioxide is a known cause of mutation in mammalian cells. Gamma tocopherol is a more potent inhibitor than \( \alpha \)-tocopherol of neoplastic transformation because of its superior ability to scavenge and chemically reduce NO\(_2\) without forming a nitrosating intermediate.

e. Gamma-Tocopherol and Its Metabolite, \( \gamma \)-CEHC, and Natriuretic Activity. The natriuretic activity is involved in several diseases including hypertension, congestive heart failure and cirrhosis of the liver (de Wardener et al., 1961; Wecher et al., 1990). It is the activity that inhibits extracellular volume expansion, which results in
sustained natriuresis, elevating plasma concentration of a Na⁺ transport inhibitor and pressor activity.

Recently, it has been shown that γ-tocopherol was efficiently metabolized to 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxy chroman (S-γ-CEHC, LLU-α), and S-γ-CEHC, which have been shown to exhibit a natriuretic activity. Moreover, it is found to be the most potent natriuretic agents among tocopherol forms and the mechanism is via inhibition of the 70 pS potassium channel in the apical membrane of the thick ascending limb of the kidney, the assumed mechanism of action of the natriuretic hormone (Chiku et al., 1984; Kantoci et al., 1997; Murray et al., 1997). Inhibition of this channel could significantly block the recycling of potassium ion and thereby, inhibit the function of Na⁺/2 Cl⁻/K⁺ cotransporter, resulting in natriuresis (Wechter et al., 1996).

Moreover, study by Murray et al., 1997 demonstrated that a metabolized of a minor component of the vitamin E complex, S-γ-CEHC, obtained only through diet, is an effector of the 70 pS potassium channel. The analogous compound derived from α-tocopherol was, on the other hand, completely inactive as a potassium channel inhibitor. The investigators further investigated the biological mechanism by which γ-tocopherol may inhibit potassium channel. Since the antioxidant potential of an α-tocopherol is greater than that of γ-tocopherol, there is increased likelihood that the chroman ring of γ-tocopherol remains intact. The proposed side-chain oxidation without chroman ring oxidized may be necessary for the production of an important regulator of the 70 pS potassium channel and possibly in the regulation of extracellular fluid volume. These findings suggested that the oxidative metabolite of γ-tocopherol, (LLU-α, S-γ-CEHC) may be the source, all or in part, of helping modulate extracellular fluid volume in
mammals. In addition, the potassium channel inhibition taken together with the lack of inhibition of sodium ion pump is consistent with the hypothesis that metabolite of \( \gamma \)-tocopherol is the putative natriuretic hormone (Takata et al., 2002).

There is a study underway to determine the concentration of LLU-\( \alpha \) in the urine of pregnant woman, congestive heart failure, cirrhosis and head trauma patients and volume-expanded states. If natriuretic activity were found, \( \gamma \)-tocopherol metabolite would be the second example of vitamin, after vitamin D, to act as a precursor for a hormone.

**f. Gamma-Tocopherol and Platelet Aggregation.** Platelet aggregation plays an important role in thrombosis and cardiovascular events (Conti et al., 1987; Handin 1996). A number of epidemiologic studies, however, have demonstrated an inverse association between CHD and vitamin E intake (Rimm et al., 1993; Stampfer et al., 1993; Kushi et al., 1996). In contrast, there was no proven association from clinical trial (Yusuf et al., 2000; GISSI 1999). Also not only is the association between tocopherols intake and inhibition of platelet aggregation are inconsistent, but also the biological mechanism by which tocopherols inhibit platelet aggregation is not completely identified.

Saldeen et al., 1999 have designed a study to examine the differential effects of \( \alpha \)- and \( \gamma \)-tocopherol on parameters of oxidation-antioxidation and thrombogenesis in rats. They found that \( \gamma \)-tocopherol supplementation led to a more potent decrease in platelet aggregation and delay of arterial thrombogenesis than did \( \alpha \)-tocopherol supplementation. Moreover, both \( \alpha \)- and \( \gamma \)-tocopherol decreased arterial superoxide anion generation, lipid peroxidation and LDL oxidation. In this experimental investigation, they concluded that a
mixed tocopherol rich in γ-tocopherol could decrease platelet aggregation and intraarterial thrombus formation in rats.

In addition, Liu et al., 2003, also reported the greater potency in preventing platelet aggregation of mixed tocopherols than α-tocopherol alone in human. In this study, participants were randomly assigned into three groups: α-tocopherol, mixed tocopherol and control for 8 weeks. This study found that mixed tocopherol has a more potent platelet inhibitory effect and that the cellular uptake of mixed tocopherol is higher than those of α-tocopherol. The biological mechanism by which tocopherols inhibit platelet aggregation is somewhat unclear. However, two of the proposed mechanism of actions were related to nitric oxide (NO) bioactivity and inactivation of cellular protein kinase C (PKC).

The first proposed biological mechanism is associated with increases in NO release, activates of endothelial constitutive NO (ecNOS) synthase. Mixed tocopherols were more potent in modulating NO release and ecNOS activation than α-tocopherol alone. One of NO mechanisms of action is to reduce platelet recruitment to a growing thrombus and inhibit platelet aggregation. Thus, reduced bioavailability of NO is an indicator in patients with CAD and impaired of platelet NO production may predict acute heart disease (Freedman et a., 1997, Freedman et al., 2000). Incorporation of tocopherol into the diet has been found to increase platelet NO concentration by preventing it from quenching by peroxyl radicals. Moreover, γ-tocopherol is the potent activator of ecNOS (Huie et al., 1993; Christen et al., 1997).

The other proposed mechanism by which tocopherols inhibit platelet aggregation is thought to be through PKC mechanism. It was reported that intake of tocopherols
resulted in a marked decrease in PKC activation by altering its phosphorylation state (Ricciarelli et al., 1998; Freedman et al., 1996).

In conclusion, mixed tocopherol may prevent platelet aggregation by increased NO release, activating ecNOS and inhibiting PKC activation in platelets. Mixed tocopherols have more potent effects on platelets aggregation than that of α-tocopherol alone.

g. Gamma-Tocopherol and Inflammation. Antioxidant vitamins, defending agents against oxidants produced during inflammation, are believed to play an important role in public health and human disease prevention. Alpha-tocopherol, the principal form of vitamin E in various tissues, have been extensively studied in vitro and \textit{in vivo}. In contrast, the data of γ-tocopherol on the anti-inflammatory are scarce to date. This is mostly due to its relatively low levels in plasma and tissue concentrations.

However, growing evidences indicates that γ-tocopherol may be essential in the defending mechanism against degenerative disease. Several investigators demonstrated the inverse correlation between γ-tocopherol and the incidence of inflammatory related diseases.

Cooney et al.1993, reported the superiority of γ-tocopherol to α-tocopherol in trapping reactive nitrogen oxide species (NO\textsubscript{x}), mutagenic electrophiles generated during inflammation. Nitrogen oxides could cause mutations in bacteria and mammalian cells due to deamination of DNA. The end products of NO\textsubscript{x} species, nitrite and nitrate, may contribute to chronic inflammation. Therefore, detoxification ability of γ-tocopherol in trapping NO\textsubscript{x} species and forming a more stable adduct, 5-nitro-γ-tocopherol, is essential in the prevention of inflammatory related diseases.
Another in vivo study demonstrated the protective effects of γ- and α-tocopherol against peroxynitrite induced lipid peroxidation. This study found that lipid hydroperoxide formation in liposomes exposed to peroxynitrite was inhibited more effectively by γ-tocopherol than α-tocopherol. In addition, γ-tocopherol acts as a trap for membrane soluble electrophilic nitrogen oxides and other electrophilic mutagens, forming stable carbon-centered adducts through the nucleophilic 5-position (Christen et al., 1997). This result supports the findings by Cooney et al. that γ-tocopherol is superior to α-tocopherol in trapping reactive nitrogen oxide species, mutagenic electrophiles generated during inflammation because of the non substituted 5-position.

In addition to γ-tocopherol reactivity toward reactive nitrogen oxide species, it appears that γ-tocopherol also plays a role in defending against inflammation-related damage. Up to date, a few studies have investigated the effects of γ-tocopherol on the inflammatory response.

Jiang et al., 2000, studied the effects of γ-tocopherol and inflammatory response in macrophages and human epithelial cells after being exposed to COX-2-preinduced cells by addition of arachidonic acids (AA). They found that γ-tocopherol inhibited the generation of prostaglandin E$_2$ (PGE$_2$), an important mediator synthesized via the cyclooxygenase -2 (COX-2) during inflammation. Moreover, the major metabolite of γ-tocopherol (γ-CEHC) also exhibited an inhibitory effect to PGE$_2$ synthesis. However, γ-tocopherol required 8 to 24 hours incubation period to cause the inhibition, whereas, γ-CEHC required only an hour for the same reaction. These findings indicated that both γ-tocopherol and its major metabolite at physiological concentrations are effective in...
inhibiting COX-2 activity in intact cells, and possess anti-inflammatory activity. This anti-inflammatory property is similar to those of NSAIDs.

In the study of carrageenan-induced inflammation in Wistar rats, administration of γ-tocopherol and γ-CEHC significantly reduced PGE$_2$ synthesis at the site of inflammation. Gamma-tocopherol also inhibits leukotriene B$_4$ (LTB$_4$), another oxidized product derived from AA through the 5-lipoxygenase–catalyzed pathway responsible for inflammatory response. In addition to the inhibitory effects on the proinflammatory eicosanoids, γ-tocopherol administration reduced inflammation-mediated damage as shown by reduced lipid peroxidation. Moreover, γ-tocopherol appears to decrease TNF-α, a key proinflammatory cytokine known to activate macrophage and provoke the inflammatory response (Jiang et al., 2003).

Collectively, γ-tocopherol inhibits proinflammatory eicosanoids (PGE$_2$ and LTB$_4$), and decreases level of an inflammatory cytokine, TNF-α, in a rat inflammation model. These results strongly suggest that γ-tocopherol shows anti-inflammatory activities and may be essential for improving human inflammatory related diseases in the future.

**h. Gamma-Tocopherol and Disease**

1. *Gamma-Tocopherol and Heart Disease.* There was limited resources on γ-tocopherol and heart disease, since most of the epidemiological evidence observed the tocopherol as a whole. Majority of the data, therefore, has been presented as vitamin E.

The correlation between consumption of fruits, vegetables and others foods containing vitamin E and heart disease has been investigated in many studies.
Consistently, coronary heart disease risk was inversely related to consumption of those foods (Knekt et al., 1994, Gaziano et al., 1995, Kushi et al., 1996). Observational studies have further indicated that consumption of vitamin E more than 100 IU a day for greater than two years may lower the rates of progression of coronary artery lesions and therefore lower the rates of coronary events (Hodis et al., 1995).

Oxidized LDL could contribute to atherogenicity by many mechanisms including increasing monocyte accumulation, reducing macrophage motility in the intima and increasing cytotoxicity. Antioxidants, for instance vitamin E, is believed to attenuate atherosclerosis by preventing oxidative modification of LDL, and increase the resistance of LDL to oxidation. Oxidized LDL is taken up more rapidly than native LDL by macrophages to create foam cells, leading cause of atherosclerosis lesions. Oxidized LDL has also been identified as endothelial cells cytotoxic and may increase vasoconstriction in arteries. The epidemiological results from the U.S. health professions both males and females provided evidence of an association between a high intake of vitamin E and a lower risk of coronary heart disease (Rimm et al., 1993, Stampfer et al., 1993).

Up to date, there have been four randomized, controlled trials of the correlation between vitamin E and coronary heart disease; however, the results are inconsistent. In a Chinese study, there was no significant reduction in cardiovascular events after 5.2 years of vitamin E supplement 30 mg daily (Blot et al., 1993). The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (ATBC), also demonstrated no effect on the risk of death from coronary heart disease of daily treatment of 50 mg of vitamin E for 5-8 years (ATBC, 1994). Consistently, the results of a recent
Italian trial demonstrated that supplemented vitamin E 300 IU daily did not alter the number of patients with myocardial infarction significantly (GISSI, 1999).

In contrast to those interventional trials, Stephens et al., 1996 reported a tremendous reduction in the number of patients with nonfatal myocardial infarction observed after receiving vitamin E 400 IU for 1.4 years. They also claimed that 400-800 IU of vitamin E supplement significantly reduced the incidence of cardiovascular death and nonfatal myocardial infarction by 77%.

Öhrovall et al., 1996 conducted a cross-sectional study of tocopherols levels in coronary heart disease patients compared to health age-matched reference participants. They reported lower mean concentration of γ-tocopherol and a higher ratio of α- to γ-tocopherol in coronary heart disease patients. These findings indicated a difference in antioxidative status between coronary heart disease patients and healthy participants.

2. Gamma-Tocopherol and Cancer. Number of scientific evidences suggested that low plasma levels of tocopherols might be used as a strong predictor for incidence of cancer disease. In addition, γ-tocopherol, is recently believed to be effective anticancer agents with low cellular toxicity and also be beneficial in human cancer disease prevention (Stone et al., 2004; Giovannucci 2000; Flohè et al., 1999). Current epidemiological and experimental studies consistently suggest that γ-tocopherol may be superior to the commonly tested, α-tocopherol, as a cancer preventive.

In a nested case-control study, Helzlsouer et al., 2000, reported that men in the highest quintile of plasma γ-tocopherol concentration had a 5-fold reduced risk of prostate cancer compared with those in the lowest quintile. They also found the statistically significant protective associations of cancer and high levels of γ-tocopherol.
Given these data, γ-tocopherol may be a better chemopreventive agent than α-tocopherol. However, the use of combined α-tocopherol and γ-tocopherol supplements for prostate cancer prevention is still recommended due to the interaction between those two tocopherol forms. The molecular mechanism behind the observed effect has not been elucidated.

To discover the molecular anticancer mechanism of γ-tocopherol, Gysin et al., 2002, studied the human prostate carcinoma and colorectal adenocarcinoma. In this study, γ-tocopherol have been reported to inhibit cell cycle progression via reduction of cyclin D1 and cyclin E levels. In addition, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman, metabolites of γ-tocopherol, shown to be more potent than α-tocopherol in inhibiting prostate cancer cell growth by the same mechanism as γ-tocopherol (Galli et al., 2004)

Jiang et al., 2004 found that γ-tocopherol exhibited antiproliferation effect on prostate cancer cells by inducing apoptosis in androgen-sensitive LNCaP. This effect of γ-tocopherol was partially reversed by the addition of arachidonic acid or linoleic acid, the substrates for cyclooxygenase and lipoxygenase.

The cohort study, the alpha-tocopherol, beta-carotene cancer prevention (ATBC), reported that participants with higher circulating concentrations of the major vitamin E fractions, α- and γ-tocopherols, had lower prostate cancer risk. In addition, incidence of prostate cancer risk was reduced by 32% in response to daily α-tocopherol supplementation (Weinstein et al., 2005).
Vitamin E and other antioxidants are believed to prevent colon cancer by decreasing the formation of mutagens generated from free radical oxidation. Therefore, Stone et al., 2004, conducted the study of chemopreventive potential of different forms of vitamin E on colon cancer cell lines in both animal and human models. They found that vitamin E levels in feces, colonocytes, plasma and liver were higher in rats fed with α-tocopherol supplementation. Moreover, compared to α-tocopherol, γ-tocopherol was found to be a more potent enhancer of proliferators-activated receptor-γ, an effective chemopreventive agents in a rat model of carcinogenesis. Similarly, Campbell et al., 2003 also reported the protective effect of γ-tocopherol by upregulating the expression of intracellular concentrations of peroxisome proliferators activated receptor-γ. This ability of γ-tocopherol in colonic tissue may be relevant to colon cancer prevention.

2. Fatty Acids

a. Fatty Acid Composition of the Diet and of Walnuts. Walnuts have a unique fatty acids composition containing ample quantities of the n-3 fatty acids, α-linolenic acids (18:3, n-3, ALA). Values per 100g edible portion are palmitic (16:0) 4.40g, stearic (18:0) 1.66g, oleic (18:1) 8.80g, linoleic (18:2) 38.10g and α-linolenic (18:3) 9.08g.

The predominant polyunsaturated fatty acids in diets, especially western diets are n-6 fatty acids. When the amount of n-3 fatty acids in the diet increases, they will replace some of the n-6 fatty acids in membranes of cells such as monocytes, lymphocytes, granulocytes and other cells.
The fatty acids of membranes serve as precursors for the *in vivo* synthesis of eicosanoids which in turn modulate inflammation and immunity. Both the level of fat in the diet and the types of fatty acids present in the diet can affect the function of lymphocytes and other immune cells.

Competition between n-6 and n-3 fatty acids occurs in prostaglandin formation. Eicosapentaenoic acid (EPA), an n-3 fatty acid, competes with arachidonic acids, an n-6 fatty acids, for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level. Studies have shown that the ingestion of fish or fish oils containing n-3 fatty acids leads to a decrease in leukotriene before formation.
Linoleic acid (18:2, n-6)

γ-Linoleic acid (18:3, n-6)

Arachidonic acid (20:4, n-6)

(+)

Proinflammatory lipid mediators
(PEG2, LTB4, TXA2)

(-)

Pharmaceutical inhibitors and mononuclear antibodies

(PEG2, LTB4, TXA2)

α-Linolenic acid (18:3, n-3)

Stearidonic acid (18:4, n-3)

Eicosatetraenoic acid (20:4, n-3)

Eicosapentaenoic acid (20:5, n-3)

Proinflammatory peptide mediators
(TNF-α, IL-1β)

Figure 3. Common molecular targets for anti-inflammatory therapies of the dietary n-3 fatty acids and pharmaceuticals.
(adapted from James et al., 2000)
b. The Immune Response and Cytokines. Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses (Turnball et al., 1999).

Cytokines are large (8-60 kDa), soluble polypeptide mediators that regulate growth, differentiation and function of many cell types in both host defense and normal and abnormal homeostatic mechanisms. Thus they have vital roles in haematopoiesis, inflammatory responses and the development and maintenance of immune responses. Furthermore, many cytokines may also induce fever, sleep, anorexia, malaise and alterations in neuroendocrine secretions (Townsend et al., 2000; Barrett 1996; Lucey et al 1996). They may be classified on the basis of their cell of origin, their spectrum of activity, the category of activity they influence, the cells that they target, or on the specific features of their ligan-receptor interaction, however, their classification remain somewhat arbitrary. Most commonly, cytokines have been classified into families of interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), chemokines, hematopoiyetin and colony-stimulating factors (CSF). As mentioned above, many cytokines are overlapping in their actions therefore they could belong to more than one family.

The difference in nature of the threat, i.e. bacterial, viral and inflammation to tissue homeostasis result in diverse production of particular cytokines response. Cytokine production increases significantly during cell disturbance. During cellular challenges such as tissue remodeling, disease, infection or trauma, cytokines are drastically produced. Not only could the nature of the threat, but also the type of tissue
being provoked and the hormone milieu possibly determines the amount and type of cytokine synthesis.

The regulation of inflammation by cytokines is intricate due to the fact that the immune system has redundant pathways with multiple elements having similar physiologic effects. For instance, one cytokine may fulfill a number of roles during inflammatory reactions. Thus typical properties of cytokines are pleiotropy, redundancy, synergistic activity and antagonistic effects upon each other (Barrett 1996; Opal et al., 2000; Townsend et al., 2000). Disturbing the cytokines regulatory network either by genetic, environment or foreign microbial may cause lethal consequences. The net effect of any cytokines, however depend on the timing of cytokine release, the location in which it acts, the presence of competing or synergistic elements, cytokine receptor density and tissue responsiveness to each cytokine (Dinarello, 1998; Turnball et al., 1999; Westendorp et al., 1997; Donnelly et al., 1996; Opal et al., 1996).

One of the physiological effects of cytokines disturbance is inflammation. It is characterized symptomatically by pain, redness and swelling and excessive inflammation entails loss of function. It is the process whereby blood cells and proteins enter tissues in response to injury, infection, trauma or immune reaction.

There are two basic forms of inflammatory cytokines, proinflammatory and anti-inflammatory. Proinflammatory cytokines include IL-1, IL-6, IL-8, TNF-α and those produced by Th1 cells (IL-2 and gamma interferon). These cytokines favor the production of inflammatory reactions. On the other hand, anti-inflammatory cytokines favor the production of immunoglobulin E and activation and production of eosinophils and mast cells. This includes IL-1 antagonist, transforming growth factor-β and those produced by
Th-2 cells (IL-4, IL-5, IL-10). An imbalance of proinflammatory and anti-inflammatory cytokines could cause inflammatory or allergic diseases (Lucey et al., 1996; Berguer et al., 1999; Johnson et al., 2003; Kelley 2001).

Inflammation mechanism has been recognized to play a central role in mediating all phases of atherosclerosis, from initial recruitment of circulating leukocytes to the arterial wall to eventual rupture of vulnerable atherosclerotic plaque. Accordingly, numbers of evidence has been compiled suggesting that markers of inflammation are strong predictors of cardiovascular diseases such as myocardial infarction and stroke (Blake et al., 2002; Pradhan et al., 2002; Hwang et al., 1997; Ridker et al., 1998).

Among the key inflammatory mediators are the n-6 eicosanoids, prostaglandin (PGE) and leukotriene (LTB). Also important are the cytokines, interleukin-1, and tumor necrosis factor (TNF). Under normal physiological circumstances, these cytokines and eicosanoids serve as immuno-modulators that confine or sustain the potential injury of inflammatory reactions. Under the pathological circumstance that these cytokines either provide ineffective control over proinflammatory activities or overcompensate and inhibit immune response, leaves the body at risk from systematic infections (Manoz et al., 1991; Kasai et al., 1997; Fitzpatrick et al., 2001).

1. **Interleukin-1β**. IL-1 includes two distinct proteins, IL-1α and IL-1β that play vital roles in acute and chronic inflammation, both locally and systematically. IL-1β is produced primarily by monocytes and macrophages, yet also by astrocytes, oligodendroglia, adrenal cortical cells, NK cells, endothelial cells, keratinocytes, megakaryocytes, platelets, neurons, neutrophils, osteoblasts, trophoblasts, T cells and fibroblasts. It elicits many biological responses including thymocyte
proliferation, fever reduction, wound healing and tissue resorption (Ito et al., 1996; Hunter et al., 1995).

In female rats, IL-1 induces a variety of behavioral, neurochemical and physiological effects including fever, suppression of food and water intake, condition of taste aversion, reduced social exploration and reduced sexual behavior (Sung et al., 2002).

IL-1β, the most potent proinflammatory cytokines, has been proved to be a potent stimulator of anxiogenic actions, and induces stress and anxiety-like behavior in rodents. This cytokine activates the hypothalamus to release corticotrophin-releasing factor (CRF), which induces the secretion of glucocorticoids from the adrenal. It also activates CNS thereby increasing the turnover of noradrenaline, serotonin and dopamine (Connor et al., 1998; Lacosta et al., 1998).

There is concrete knowledge that the immune system and CNS form a complex interacting network. It is certain that there is a communication between the aspects of immune system, endocrine system and CNS. Thus immune activation may provoke neuroendocrine and central nervous system (CNS). In fact activating the immune system may affect endocrine and transmitter processes and may influence some of the behavioral effects (Song et al., 1999; Elenkov et al., 2000; Song 2000; Maier et al., 1998).

Studies of the immune response effects on cognitive function discovered that induction of IL-1β might cause brain inflammation and lead to cognitive impairment. In patients with Alzheimer’s disease and other neurodegenerative diseases, increase in IL-1β and other proinflammatory cytokines have been associated with cognitive impairment (Licastro et al., 2000; Kalaria et al., 1996).
Interleukin-1 is involved in activation of hypothalamus-pituitary-adrenal (HPA) axis and neurotransmitter in several studies. Increasing secretion of corticosterone, reduced release of acetylcholine and increasing metabolites of serotonin and dopamine in the hippocampus and other limbic regions of the brain are related to IL-1 concentration. In addition, systemic administration of IL-1β could induce brain inflammatory response and the expression of amyloid precursor protein (Oitzl et al., 1993; Gibertini et al., 1995; Potter et al., 2001; Dunn 2000).

Even though concentrations of IL-1 is strongly related to cognitive impairment, the most extensive study about the IL-1 is associated to their potent initiation of inflammation. The physiological action of IL-1 is subsequent to various cells being attacked by bacterial endotoxin or varieties of non-microbial inflammatory substances. After threatening actions, IL-1 would be released with excessive quantities to the circulation and trigger a general inflammatory response.

In a general inflammatory response, circulating IL-1 could function by inducing capillary endothelial cells to secrete chemokines and increasing expression of cell adhesion molecules (i.e. E-selectin, ICAM-1 and VCAM-1). Those chemokines and adhesion molecules would further activate mononuclear cell integrins and facilitate mononuclear infiltration into the area. Together with IL-2, IL-1 induces NK cells to release interferon (IFN-γ), resulting in IFN-γ-induced activation of macrophages (Hunter et al., 1995).

In addition, IL-1 regulated matrix metalloproteinases (MMPs), which could initiate extracellular matrix degradation, monocytes migration, embryo implantation, tissue involution, angiogenesis and wound healing. It is generally accepted that elevated
levels of IL-1 is the key mediator that enhances the biosynthesis and secretion of MMPs. Thus, the promotion of wound healing and tissue degradation is considered to be in part due to IL-1 stimulated cells. Thus, suppression of IL-1 synthesis and activity is the most effective strategy to control physiological inflammatory response (Ito et al., 1996; Sica et al., 1990).

2. Interleukin-6. IL-6, like IL-1, is a proinflammatory cytokine released from activated macrophages. It has, however, less extensive function in terms of behavior, neurotransmitter and endocrine activity than those involving in IL-1. IL-6 was originally identified as a B cell differentiation factor (Akira et al., 1990).

IL-6 is a multifunctional protein synthesized by lymphoid and non-lymphoid cells. It could also be generated by normal and transformed cells including T cells, monocytes, macrophages, fibroblasts, hepatocytes, vascular endothelial cells, cardiac myxomas, bladder cell carcinomas and myelomas. And because of its various functions, IL-6 has also been known as IFN-β2, B-cell stimulatory factor-2 (BSF-2), hybridoma/plasmacytoma growth factor and macrophage-granulocyte inducing factor 2A (MGA-2A). It could be regulated either positively or negatively by a variety of signals and other cytokines including mitogens, antigenic stimulation, lipopolysaccharides, IL-1, TNF, and viruses (Breen et al., 1990; Hirano et al., 1984; Honda et al., 1992).

The effects of IL-6 on different cells are abundant and diverse. On B-cells, it encourages the differentiation and secretion of antibody. It also acts as a co-stimulant triggering IL-2 synthesis and IL-2 receptor expression (Honda et al., 1990; Kikutani et al., 1985). In addition, it also exhibits growth factor activity for mature thymic or peripheral T-cells and enhances the differentiation of cytotoxic T-cells in the presence of
IL-2 or IFN-γ. On hepatocytes, IL-6 enhances production of acute phase proteins and stimulates activity of hemopoietic stem cells. Additional bioactivities attributed to IL-6 include: inhibition of the growth and induction of terminal differentiation of myeloid leukemic cells, induction of neuronal cell differentiation and induction of the maturation of megakaryocytes (Haegeman et al., 1986; Okada et al., 1983; Kikutani et al., 1985). The various physiological effects of IL-6 mentioned above suggest that it has a major role in the mediation of the inflammatory and immune responses initiated by infection and injury (Kurzrock et al., 1993; Akira et al., 1990; Liu et al., 1992).

3. Tumor Necrosis Factor-α. TNF-α is also known as cachectin, postulated to mediate wasting during chronic infections. It is produced by activated T and B lymphocytes, neutrophils, astrocytes endothelial cells, smooth muscle cells and some other transformed cells. For the last decade its bioactivities have been investigated by numerous reviews. Most of those reviews propose the ability of activate multiple signal transduction pathways to induce or suppress the expression of a vast number of genes, including those for growth factors and cytokines, transcription factors, inflammatory mediators and acute phase proteins (Vilcek et al., 1991; Aggarwal et al., 1984; Seckinger et al., 1989; Narachi et al., 1987).

Engelmann et al., 1990 reported its effect on cells functioning in a number of ways including defending against infectious agents and recovering from injury. Thus one of the critical roles of TNF-α is to support normal host defense against infections and against growth of malignant tumors. In particular circumstance, some of these effects may become unfavorable, causing more harm than the pathogen itself. Seckinger et al., 1989 stated that TNF-α could cause hemorrhagic necrosis of the tumors and cytotoxic effect in
certain cell lines during pathological stage. In addition, it stimulates production of IL-1, with which it share several biological activities. Together with IL-1, it could promote collagenase production of human dermal fibroblasts and induce fever by stimulating hypothalamic prostaglandins synthesis. Thus many studies projected that many of its bioactivities functionally resemble the effects produced by IL-1.

Vilcek et al., 1991 reported that elevated levels of TNF-α could affect a number of pathological conditions including cachexia, septic shock following gram negative bacteria and autoimmune disorders. In fact, its overproduction could lead to severe systematic toxicity and even death. It also has been implicated in the pathogenesis of some autoimmune disorders and of graft-versus host defense (Aggarwal et al., 1984; Sica et al., 1990).

4. **E-Selectin.** The selectin family consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin) and vascular endothelium (E and P-selectin). The selectins function is restricted to leukocyte interactions with vascular endothelium (Dong et al., 1998; McEver 1994). P and E selectins are adhesion molecules mediating the first step in leukocyte extravasations.

At the site of tissue injury and inflammation, selectin mediates the initial attachment of leukocytes to venular endothelial cells before their firm adhesion. Multiple studies indicated that selectins mediate neutrophil, monocyte and lymphocyte rolling along the venular wall (Lobb et al., 1991; Weller et al., 1991; Gearing et al., 1992).

E-selectin also known as endothelial Leukocyte Adhesion Molecule-1, expressed only on endothelial cells and only after activation by inflammatory cytokines (IL-1β,
TNF-α) or endotoxin (Bevilacqua et al., 1993; Kansas 1996). Like other selectins, E-selectin is a mediator of the rolling attachment of leukocytes to the endothelial cells, an essential step in extravasation of leukocytes at the site of inflammation, thereby playing a major role in localized inflammatory response. Expression of E-selectin is transitory, reaching a maximum within approximately six hours of stimulation and then declining with shedding of soluble E-selectin in circulation (Guray et al., 2004; Kansas 1996; Gearing et al., 1992). Elevated level of E-selectin in serum has been reported in a variety of pathological conditions.

Up to date, there is increasing evidence that suggests that E-selectin contributes to tumor growth and metastasis. It may promote tumoral angiogenesis and the adhesion of tumoral cells in endothelial cells at distant sites (Makker et al., 2002; Hebbar et al., 1998).

5. **C-Reactive Protein.** C-reactive protein (CRP) is an acute phase protein with a well known association with infection and other inflammatory conditions. It is the prototype of acute-phase proteins in humans and is widely used as a gauge for the presence and extent of systematic inflammation (Ridker et al., 1998; Lopez et al., 2004; Ridger et al., 2002).

The normal serum level of CRP is approximately less than 1μg/ml yet can be elevated as much as 1000 fold within 2-3 days after onset of acute inflammation (Torzewski et al., 2000; Heuertz et al., 1994). The fact that it could be dramatically increased and highly conserved throughout evaluation indicates its major physiological function.
CRP is essential in modulating a number of inflammatory and immune responses and localizes to sites of inflammation in association with neutrophils. Study has shown that CRP inhibits neutrophil chemotaxis to C5a in vitro (Robey et al., 1987). The chemotaxis effect of CRP on monocyte/macrophage however is controversial. One report demonstrated the effect of CRP on the stimulation of human monocyte chemotaxis and procoagulant activity (Whisler et al., 1986). Nevertheless, another study has shown that no effect on monocyte chemotaxis (Robey et al., 1987).

**c. Fatty Acids and the Immune Response.** Simopoulos 2002 reported that, 20-25 fold more of n-6 fats than n-3 fats were consumed in the typical western diet. According to Continuing Survey of Foods Intakes by Individuals, CSFII (1994-1996), however, reported current US dietary intake of linoleic acids, LA (18:2, n-6) to α-linolenic acids, ALA (18:3, n-3) is 9.5:1, where as the current recommendation is 5:1. This predomiance of n-6 fats is suspected due to their abundant proportion in the diet.

N-3 PUFA is believed to be potent modulators of lymphocyte, monocyte, macrophage, neutrophil and endothelial function and thus modulates immune and inflammatory responses. Thus, in the past 15 year, many studies have investigated the effect of n-3 PUFA on human immune and inflammatory responses. Simopoulos 2002, reported that among the fatty acids, n-3 PUFAs is the most potent immuno-modulator. In fact, n-3 PUFA modulates the amount and type of eicosanoids made and controls intracellular signaling pathways, transcription factor activity and gene expression.

The effect of fish oil supplementation on the production of monocyte cytokines was examined in a number of studies. Such studies have shown that n-3 PUFA can
diminish peripheral blood mononuclear cell proliferation and reduce the production of IL-2, IL-1, IL-6, TNF-α and interferon.

In 1989, Endres et al., investigated the influences of n-3 PUFA on production of TNF-α and IL-1β. Nine healthy volunteers incorporated fish oil concentrate into their diet for six months. Production of IL-1β and TNF-α were inhibited by 40% and 61% respectively.

Meydani et al., 1991 reported that n-3 PUFA supplementation inhibited proinflammatory cytokines synthesis in young and old women differently. There were 48% and 90% inhibition of IL-1β synthesis in young and older women respectively. There was 58% inhibition of TNF-α synthesis in young and 70% in older women. The study further concluded that n-3 PUFA supplementation reduced cytokine production in young women but inhibited T cells mitogenesis in older women. Caughey et al., 1996 studied the effect of n-3 PUFA on TNF-α and IL-1β synthesis in twenty eight healthy volunteers. For the first four weeks of a flaxseed oil diet consumption, TNF-α and IL-1β synthesis were inhibited by approximately 30%. Fish oil diet continued for another four weeks, inhibited TNF-α and IL-1β synthesis by 74% and 80% respectively. They concluded that vegetable oil could also demonstrate inhibition of proinflammatory cytokines yet in lesser extent. Table 4.

In a cross-sectional study of 727 women from the Nurses’ Health Study, n-3 PUFA was inversely associated with the levels of IL-6 in healthy women. IL-6 level was 23% lower among those in the highest quintile of n-3 PUFA compared with the lowest quintile (Garcia et al., 2004).
Table 4. The Inhibition of IL-1, IL-6, and TNF-α Production After n-3 PUFA Consumption

<table>
<thead>
<tr>
<th>References</th>
<th>Subjects</th>
<th>Weeks of consumption</th>
<th>Daily amount of n-3 PUFA consumption</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caughey et al., 1996</td>
<td>28 healthy</td>
<td>8</td>
<td>1.62 g EPA, 1.08 g DHA</td>
<td>78-81</td>
</tr>
<tr>
<td>Meydani et al., 1991</td>
<td>6 healthy (23-33y)</td>
<td>12</td>
<td>1.68 g EPA, 0.72 g DHA</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>6 healthy (51-68y)</td>
<td>12</td>
<td>1.68 g EPA, 0.72 g DHA</td>
<td>90</td>
</tr>
<tr>
<td>Endres et al., 1989</td>
<td>9 healthy</td>
<td>6</td>
<td>2.7 g EPA, 1.8 g DHA</td>
<td>61</td>
</tr>
<tr>
<td>Kremer et al., 1990</td>
<td>20 RA patients</td>
<td>24</td>
<td>27 mg EPA, 18 mg DHA/kg</td>
<td>40.6</td>
</tr>
<tr>
<td></td>
<td>17 RA patients</td>
<td>24</td>
<td>54 mg EPA, 36 mg DHA/kg</td>
<td>n/a</td>
</tr>
</tbody>
</table>

(James et al., 2000)
In a double-blind cross-over study of hypercholesterolemia individuals, IL-6 and TNF-α were significantly higher after consumption of a stick margarine diet compared to a soybean oil diet. The ratio of total cholesterol to HDL-C was positively related to IL-1β and TNF-α production. The study concluded that a diet high in hydrogenated fat increases inflammatory cytokines production and may lead to pathophysiology of atherosclerosis (Han et al., 2002).

In animal studies, feeding laboratory animals a fish oil diet also resulted in markedly decreased production of IL-1, IL-6 and TNF-α by inflammatory macrophages. Song et al., 2003 evaluated the effect of an n-3 fatty acids (EPA) supplemented diet on the stress/anxiety behavior in rats, which were induced by IL-1β administration. They discovered that EPA supplemented diet could reduce the elevated prostaglandins E2 secretion and increased secretion of IL-10. In addition, an EPA supplemented diet attenuated IL-1β function of reducing growth rate to a great extent. They concluded that EPA supplemented diet appears to antagonize the endocrine, immune, and behavioral effects of IL-1β administration.

In 2004 Song et al., compared the inflammation-induced impairment of spatial memory in rats consuming coconut oil, soybean oil and EPA diets. After administering of IL-1, serum corticosterone was increased, and spatial memory deficits were observed. IL-1β also caused an increase in the hippocampal prostaglandin E2 concentration. These effects of Il-1 administration were attenuated in the group of rats that consumed the EPA diet.

Foitzik et al., 2002 have studied the effects of n-3 fatty acid supplemented diet on inflammatory cytokines in laboratory animal induced pancreatitis. They reported that
infusion of n-3 fatty acids increases anti-inflammatory cytokines. In fact EPA-derived mediators generated from n-3 fatty acids were less active or even exhibit anti-inflammatory effects in pancreatitis.

It is believed that induction in endothelial cells of adhesion molecules for circulating leukocytes and of inflammatory mediators by cytokines probably contributes to the early phases of inflammation. The proposed mechanisms by which dietary fatty acids can modulate inflammation however are limited.

Yasunori et al., 1998 investigated the effect of triglyceride reduction on cell adhesion molecules (sCAM) levels and E-selectin in twenty seven diabetic patients. To test the hypothesis that triglyceride could modulate e-selectin concentration, n-3 fatty acids (Omacor) have been given to patients at 4 g/d. After 7 months of supplementation, the results show that triglyceride level was reduced by 47 ± 4.6% and soluble E-selectin level was decreased by 16±3.2%. The researchers, thus, concluded that triglycerides and HDL metabolism influence CAM expression. In addition, treatment with n-3 fatty acids may alter vascular cell activation through inhibition of adhesion of monocytes.

Incorporation of DHA into cellular lipids may decrease expression of endothelial leukocyte adhesion molecules, secretion of inflammatory mediators and leukocyte adhesion to cultured endothelial cells. In fact, DHA controlled cytokine-stimulated endothelial cell expression of E-selectin (Caterina et al., 1994).

In addition, in 727 women from Nurses’ Health Study I, the intake of ALA was inversely associated with plasma concentrations of E-selectin (β=-0.24) after controlling for age, BMI, physical activity, smoking status, alcohol consumption and intake of LA.
E-selectin level was 10% lower among those in the highest quintile of total n-3 PUFA consumption, compared with the lowest quintile (Garcia et al., 2004).

In 2003, Pischon investigated habitual n-3 PUFA intake and its interaction with n-6 PUFA in relation to the plasma inflammatory marker CRP among healthy 405 men and 454 women. The results show that after adjusting for other predictors of inflammation, intake of EPA and DHA was somewhat inversely associated with plasma levels of CRP.

In a cross-sectional study of 727 women from Nurses’ Health Study, CRP level was 29% lower among those in highest quintile of total n-3 PUFA, compared with the lowest quintile. In fact, after adjusting for age, BMI, physical activity, smoking status, alcohol consumption and intake of LA, the intake of ALA was inversely related to plasma concentration of CRP ($\beta = -0.55$) (Garcia et al., 2004).

Besides consumption of n-3 PUFA, dietary fiber also influences plasma level of CRP. Ajani et al., 2004 examined the association between dietary fiber and serum concentration of CRP using data from the National Health and Nutrition Examination Survey 1999-2000. The odds ratio (OR) for increased CRP concentration was 0.49 for the highest quintile of fiber intake compared with the lowest intake.

Moreover, a randomized crossover design, compared the effect of trans fatty acid to carbohydrate on CRP concentrations. Eight percent of fat was replaced by trans fatty acids in diet of 50 men. After a 5 week period, CRP concentration was not statistically significantly higher after consumption of the trans fatty acids than after consumption of the carbohydrate diet (Baer et al., 2004).
In conclusion, n-3 fatty acids in the diet can suppress the production of inflammatory mediators including TNF-α, IL-1β and IL-6. The mechanism responsible for the suppression of these proinflammatory cytokines production by n-3 fatty acids remains unclear, although suppression of eicosanoid production may be involved. Many anti-inflammatory pharmacotherapies are intended for inhibiting the production of these cytokines thus incorporate n-3 dietary fatty acids to diet may be beneficial.
A. Subjects and Recruitment

Subjects for the study were individuals selected from those who participated in the Walnuts Feeding Trial conducted by Joan Sabaté and colleagues at the Department of Nutrition, School of Public Health, Loma Linda University.

Subjects for the Walnuts Feeding Trial were recruited from Loma Linda community and neighboring regions. Local publications, flyers and radio were utilized for recruitment purposes at local companies and institutions. Subjects were recruited by a multi-stage process. First, interested persons completed a preliminary questionnaire via phone or email. Second, select group was invited to a group informational meeting. During the meeting, purposes, procedure and requirement of the study were explained to the potential subjects. Third, those interested in participating were further interviewed individually following the meeting. Each subject received an explanation and signed an informed consent before filling out medical and lifestyle questionnaires.

All eligible subjects were scheduled to classify information and to evaluate perception of compliance for metabolic feeding study. Distance from Loma Linda University, work schedule and reason of interest in the studied were investigated. The study was approved by the Institutional Review Board of Loma Linda University.

Admission into the study was based on following medical/biological criteria. Subjects between the ages of 30-70, with no significant weight changes during previous six months, not over or under weight, non-diabetic, and no known allergy to nuts were considered. Subjects were also required to have their habitual diet closely related to the
typical American diet with no nut consumption less than once per week. One hundred and eleven subjects began the study. Ninety completed the trial.

Eighty subjects who had good compliance to their diet and who had their blood draw completed at 4, 6, 10 and 12 month were selected for tocopherol analysis.

Thirty subjects who had the highest compliance to their diet by incorporating walnuts into their diet more than 10% of daily energy intake according to the dietary recall data, no anti inflammatory or other related medication use reported were selected for proinflammatory markers analysis.

B. Protocol

The Walnut Feeding Trial was a 12-month randomized crossover intervention study, which consisted of two phases, treatment and control. Ninety healthy adults participated in this study. They were randomly assigned to either of these two phases for the first six months and then switched to the other phase for the second six months. During the treatment phase, participants volunteered to incorporate a portion of walnuts equal to 12% of their daily energy intake (range 28 grams to 56 grams per day) in their habitual diet. During the control phase, they followed their habitual diet. No other dietary advice was given to either group.

C. Study Diet

Subjects were randomly assigned to start with either habitual diet (control phase) or walnut supplemented diet (treatment phase). During control phase, subjects consumed their usual diet for six month. Subjects were instructed to consume walnuts equal to 12% of daily energy intake per day for six months for treatment phase. Walnuts were provided
at time of clinic visits. No instructions as to time of the day or whether included in cooked food were concerned.
Figure 1. The study design
Note:
Habitual diet: usual diet
Walnuts diet: habitual diet + walnuts 12% of energy intake
D. Data Collection

Subjects were required to maintain their activities, exercise and other lifestyle habits. Any signs of illness experienced and, or supplements or medications ingested were recorded by the investigators.

Blood specimens of each participant were obtained periodically at 4, 6, 10 and 12 months. The process of venipuncture followed the standard protocols of blood clinics. The blood specimens were stored frozen at -80 degree Celsius until analysis at the Nutrition Laboratory, Department of Nutrition, School of Public Health.

1. Plasma Tocopherols Analysis

Plasma samples were examined for tocopherols by High Performance Liquid Chromatography (HPLC) method, utilizing a Normal Phase diol column as described by Kramer et al., 1997. Solvents were HPLC grade isopropyl alcohol and hexane UV. The tocopherols: alpha, beta, gamma, and delta were used as standards. The extraction of tocopherols consist of pipetting 400 microliters of plasma and 400 microliters of ethanol, mix for 30 seconds. Four hundred microliters of hexane containing 4 microgram per milliliter of internal standard and 600 microliters of hexane containing BHT were added, vortexed for 2 minutes and then centrifuged for 10 minutes at 2800 rpm. The hexane layers were removed (about 600 microliters) and evaporated to dryness.

Samples were reconstituted with 200 microliters of hexane, mixed well and then analyzed for tocopherol fractions. Ten microliters of samples were injected, and then run for a 20-minute cycle. Serum samples were tested in duplicate and averages were taken for alpha, beta, gamma and delta tocopherol fractions.
2. **Plasma Inflammatory Mediators Analysis**

Plasma concentrations of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and sE-Selectin were analyzed by utilizing the quantitative high sensitivity technique in kit form according to the supplier’s instructions (R&D Systems, Minneapolis, MN). C-Reactive Protein (CRP) was analyzed by utilizing the ACTIVE US CRP Enzyme-Linked Immunosorbent Assay (ELISA) kit form according to the supplier’s instructions (Diagnostic Systems Laboratories, Inc., Webster, TX).

The absorbances of IL-1β, IL-6, TNF-α, sE-selectin and CRP (measured in optical density units) were determined with an automated spectrophotometer plate reader. The absorbance, then were directly proportional to the amount of cytokine in the standard or sample, and be converted to concentration by utilizing a standard curve.

**a. Interleukin-1β.** The quantitative determination of human IL-1β concentrations in plasma employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-1β has been pre-coated onto a microplate. Standards and samples were pipetted into the wells in duplicate and any IL-1β present was bound by the immobilized antibody. After washing any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IL-1β bound in the initial step. The color development was stopped and the intensity of the color was measured with an automated spectrophotometer plate reader set at 450nm. (R&D Systems, Minneapolis, MN)
b. Interleukin-6. The quantitative determination of human IL-6 concentrations in plasma employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells in duplicate and any IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-like polyclonal antibody specific for IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development was stopped and the intensity of the color was measured with an automate spectrophotometer plate reader set at 450nm. (R&D Systems, Minneapolis, MN)

c. Tumor Necrosis Factor-α. The quantitative determination of human TNF-α in plasma employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples were pipetted into the wells in duplicate and any TNF-α present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-like polyclonal antibody specific for TNF-α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and color developed in proportion to the amount of TNF-α bound in the initial step. The color development was stopped and the intensity of the color was measured with an automated spectrophotometer plate reader set at 490nm. (R&D Systems, Minneapolis, MN)
d. E-Selectin. The quantitative determination of human soluble E-selectin in plasma employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sE-selectin has been pre-coated onto a microplate. Standards, samples, controls and conjugate were pipetted into the wells in duplicate and any sE-selectin present was sandwiched by the immobilized antibody and a second enzyme-linked monoclonal antibody specific for sE-selectin. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of sE-selectin bound. The color development was stopped and the intensity of the color was measured with an automated spectrophotometer plate reader set at 450nm. (R&D Systems, Minneapolis, MN)

e. C-Reactive Protein. The determination of CRP in plasma employs the ACTIVE US CRP ELISA. This is an enzymatically amplified “two-step” sandwich-type immunoassay. Standards, controls and samples were incubated in duplicate in micropipette wells which had been coated with anti-US CRP antibody. After incubation and washing, the wells were treated with another anti US CRP detection antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells were incubated with substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm.

The absorbance measured was directly proportional to the concentration of US CRP present. A set of US CRP standards was used to plot a standard curve of absorbance
versus US CRP concentration from which the US CRP concentrations in the specimens can be calculated. (Diagnostic Systems Laboratory, Inc., Webster TX)

E. Statistical Analyses

1. *Plasma Tocopherols and C-Reactive Protein*

Statistical analyses were performed with SAS software for windows (version 8; SAS Institute, Cary NC). Descriptive statistics are expressed as means, and standard deviations. Blood tocopherol and CRP data were normalized by log-transformation prior to analysis. Dietary treatments effects on blood cholesterol, tocopherol and CRP variables were estimated by using mixed linear models, which included a random factor for the subjects, a fixed factor for the diet period, and a fixed factor representing the diet.

Univariate relations between log-CRP on the control diet, body mass index (BMI), gender, usage of non steroids anti-inflammatory drugs (NSAIDs), usage of lipid lowering medications and usage of vitamin E supplements were analyzed by a mixed linear (variance components) model which included a fixed factor for the subject characteristics of interest and a random factor for the subject. For this analysis data from the 4th and 6th month of the habitual diet were treated as replicate. To test the interaction between diet, vitamin E supplement usage, plasma ratios of tocopherols to cholesterol, and CRP concentrations, a main effect term for α- or γ- tocopherol to cholesterol ratio and a term for interaction between α- or γ- tocopherol and diet were added to the model. The α- and γ- tocopherol to cholesterol ratios were treated as dichotomous variables based on the median on the control diet.
Results from all mixed linear models are presented in original units after back transformation, and are presented as least-square geometric means, % difference and 95% confidence intervals.

Multiple linear regression was used to examine the combined effect of dietary plus supplemental α-tocopherol, dietary γ-tocopherol, gender, age, BMI and usage of NSAIDs in subjects on their habitual diets and on the walnut-supplemented diet. Models were constructed using stepwise variables selection and Mellows’ C criterion. Both procedure led to the same models.

2. *Plasma Inflammatory Mediators*

The effect of walnuts on proinflammatory cytokines including E-Selectin, IL-1β, IL-6, and TNF-α was examined by mixed-effect models, with fixed-effect terms for diet and period. Although the data was correlated, the correlations did not markedly decline over time, and therefore the covariance structures were modeled using compound symmetry model. In addition, gender-diet interactions were tested by adding appropriate fixed terms to the model. Correlation analysis was employed to check for possible relations between each of the proinflammatory cytokines and serum tocopherols. All outcome and tocopherol values were normalized by log transformation prior to analysis. However, for ease of interpretation, final results were presented in original units.
CHAPTER 4

TOCOPHEROLS PUBLISHABLE PAPER

The Effects of Long Term Walnuts Consumption on Plasma Tocopherols and C-Reactive Protein in Healthy Individuals

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Introduction

The results of epidemiological studies have shown a correlation between nut consumption and reduced incidence of cardiovascular disease (1-3). Several clinical trials have shown that the consumption of nuts has favorable effects on serum lipid profiles with a decrease in total- and low density lipoprotein (LDL) – cholesterol as well as triglycerides (4-6). These clinical findings explain only part of the cardiovascular disease (CVD) risk reduction observed in the epidemiologic outcomes, suggesting that nuts might have antiatherosclerotic effects beyond blood lipid lowering.

For years the main cause of atherosclerosis was assumed to be the accumulation of lipids in the arterial wall. More recently, a body of evidence has been compiled which suggests that markers of inflammation are strong predictors of cardiovascular events including stroke, peripheral artery disease and myocardial infarction (7-10). These markers include proinflammatory cytokines and acute phase proteins especially C-reactive protein (CRP). Dietary factors that affect low-level systemic inflammation are those that inhibit lipid oxidation such as tocopherols, and vitamin C and the n-3 polyunsaturated fatty acids (n-3 PUFA) (11-14).

Walnuts are a rich source of \( \gamma \)-tocopherol (20.83 mg/ 100 g edible portion), which is an important lipophilic antioxidant. In cell systems and animal studies, \( \gamma \)-tocopherol was more potent than \( \alpha \)-tocopherol with respect to inhibition of cyclooxygenase and reduction of eicosanoids and inflammatory damage (15-17).

Walnuts are one of the few plant foods which contain high concentrations of the n-3 PUFA, \( \alpha \)-linolenic acid (ALA). The amounts of ALA in a handful of walnuts (28 g)
is approximately 5 times the daily intake in the US diet. N-3 PUFA have been shown to have anti-inflammatory and antiatherogenic effects (18-19).

The aim of this present study was to determine whether concentrations of α-, γ-tocopherols and CRP in plasma are affected by the addition of walnuts to the diet of healthy individuals. We also investigated the association between walnuts intake, vitamin E supplement use and CRP levels.

Subjects and Methods

Study Population

This study was conducted in 80 healthy persons (33 men and 47 women) aged 30-72y recruited from employees of Loma Linda University and residents of the surrounding community through payroll inserts, flyers, and newspaper advertisements. The basis for this work was a study designed to determine the effect of a daily walnut supplement on body weight and composition. The study protocol, consent form, and subject-related materials were approved by the Institutional Review Board of Loma Linda University. Written informed consent was obtained from all subjects.

Eligibility criteria were that participants had to have no significant weight changes during the previous six months, a BMI<35, a habitual diet close to the typical American diet, and infrequent nut consumption (i.e., less than once a week). Excluded were individuals with a diagnosed metabolic disorder that could affect body weight (i.e., diabetes, hypothyroidism) and those with an aversion to or a known allergy to nuts. Except for fish oil and n-3 polyunsaturated fatty acid containing supplements, subjects were not required to discontinue taking their usual medications and dietary supplements. Subjects were asked to maintain their usual activities and lifestyle habits.
An initial structured interview with each participant provided information about usual medication and dietary supplement use. This information was validated by data obtained through the periodic 24-hour dietary recalls which were conducted throughout the study.

**Experimental Protocol**

The study was a randomized crossover field trial which lasted for 1 year and included two 6-month diet periods: the habitual diet of the participants; and the habitual diet plus a walnut supplement. At baseline, participants were randomly assigned to one of two treatment sequences. In the first sequence participants consumed their habitual diet plus a walnut supplement for six consecutive months then switched to their habitual diet for six consecutive months; and, in the second sequence participants started out with their habitual diet for six consecutive months and then switched to the walnut-supplemented diet for six consecutive months. **Figure 1**

**Study Diet**

The walnut supplement consisted of a quantity of walnuts that provided approximately 12% of the daily energy intake. Initial allotment of walnuts was based on projected daily energy expenditure computed using the World Health Organization’s published equations. In subsequent clinic visits, the walnut allotment was adjusted based on the actual daily energy intake as reported by the participants in the 24-hour recalls. Shelled walnuts were provided in labeled packets, one for each day of the week in amounts varying from 28g to 56g and distributed every two months. Subjects were requested to return any unconsumed portion.
Dietary Recalls

To ascertain compliance with study requirements 24-hour dietary recalls were obtained through telephone interviews by trained nutritionists using the Nutrition Data Systems for Research (Nutrition Coordinating Center, University of Minnesota, 2000). Telephone interviews were unannounced and obtained on non-consecutive days. Seven recalls were collected during each diet period for a total of 14 recalls for the study. To capture daily variations in intake, 2 weekend days and five weekdays were covered by the seven recalls.

Laboratory Analyses

Fasting (12 hour) blood specimens were obtained at baseline and at the end of months 4, 6, 10 and 12. After an overnight fast, blood was collected by venipuncture into evacuated tubes with heparin as anticoagulant. Blood specimens were centrifuged at 1200 x g at 4° C for 10 minutes, portioned and stored at -80° C for subsequent analysis.

Blood lipids were measured by conventional enzymatic methods. Plasma tocopherol concentrations were measured by high performance liquid chromatography (HPLC) using a normal phase diol column (Supelco, Bellefonte, PA) and the method of Kramer et al (19) C-reactive protein was measured by a commercial high sensitivity ELISA kit (Diagnostic Systems Laboratories, Webster, TX).

Statistical Analyses

Statistical analyses were performed with SAS software for windows (version 8; SAS Institute, Cary NC). Descriptive statistics are expressed as means, and standard deviations. Blood tocopherol and CRP data were normalized by log-transformation prior to analysis. Dietary treatments effects on blood cholesterol, tocopherol and CRP variables
were estimated by using mixed linear models, which included a random factor for the subjects, a fixed factor for the diet period, and a fixed factor representing the diet.

Univariate relations between log-CRP on the control diet, body mass index (BMI), gender, usage of non steroids anti-inflammatory drugs (NSAIDs), usage of lipid lowering medications and usage of vitamin E supplements were analyzed by a mixed linear (variance components) model which included a fixed factor for the subject characteristics of interest and a random factor for the subject. For this analysis data from the 4th and 6th month of the habitual diet were treated as replicate. To test the interaction between diet, vitamin E supplement usage, plasma ratios of tocopherols to cholesterol, and CRP concentrations, a main effect term for α- or γ- tocopherol to cholesterol ratio and a term for interaction between α- or γ- tocopherol and diet were added to the model. The α- and γ- tocopherol to cholesterol ratios were treated as dichotomous variables based on the median on the control diet.

Results from all mixed linear models are presented in original units after back transformation, and are presented as least-square geometric means, % difference and 95% confidence intervals.

Multiple linear regression was used to examine the combined effect of dietary plus supplemental α-tocopherol, dietary γ-tocopherol, gender, age, BMI and usage of NSAIDs in subjects on their habitual diets and on the walnut-supplemented diet. Models were constructed using stepwise variables selection and Mellows’ C criterion. Both procedure led to the same models.
Results

Baseline characteristics of study participants are shown in Table 1. Use of vitamin E supplements was common among the study participants with 21 individuals taking a separate vitamin E supplement, this not including those taking multivitamin-minerals supplements which contain lower doses of the vitamin.

As shown in Table 2, total cholesterol levels were significantly lower on the walnut supplemented diet in all subjects and in users of vitamin E supplements. Although the absolute concentrations of \( \alpha \)-tocopherol in plasma were lower on the walnut diet, the lipid-adjusted molar ratio of \( \alpha \)-tocopherol to total cholesterol did not change. The molar ratio of \( \gamma \)-tocopherol to cholesterol was significantly higher on the walnut supplemented diet. Mean CRP levels did not significantly differ on the walnut diet in those who did not use vitamin E supplements. However, there was a significant interaction between CRP concentrations, use of vitamin E supplements and the walnut supplemented diet. Blood CRP concentrations were substantially lower among users of vitamin E supplements and the levels increased on the walnut diet in this subgroup.

Table 3 presents the results of the univariate models. CRP was significantly lower for users of NSAIDs and vitamin E supplements than for non-users. CRP was non-significantly higher in females than in males and non-significantly lower in users of lipid lowering drugs than in non-users with only 3 participants in this category. There was no relation between age and CRP, but BMI was linearly related to \( \log(\text{CRP}) \) (\( \beta=0.088 \pm 0.029, p=0.002 \)) as shown in Figure 2.

The interaction between the walnut-supplemented diet, blood levels of \( \alpha \)-tocopherol and \( \gamma \)-tocopherol and CRP are shown in Figures 3 and 4. Exploratory
analysis indicated no relation between absolute amounts of \( \alpha \)- or \( \gamma \)-tocopherol to CRP levels. However, there was an interaction between the ratios of these nutrients to total blood cholesterol level and CRP concentration. The ratios of \( \alpha \)- and \( \gamma \)-tocopherol to cholesterol were treated as dichotomous variables based on the median of the control diet. CRP levels were significantly higher on the walnut-supplemented diet but only among those with a high ratio of \( \alpha \)-tocopherol to cholesterol and a low ratio of \( \gamma \)-tocopherol to cholesterol.

Results of the multiple linear regression analysis are shown in Table 4. During both on the habitual diet and the walnut-supplemented diet, BMI was positively associated with, and dietary \( \alpha \)-tocopherol from food and supplements, dietary \( \gamma \)-tocopherol, and use of NSAIDs were negatively associated with blood CRP concentrations.

**Discussion**

In this 12-month randomized crossover field trial we examined the effects of daily walnut intake on plasma tocopherol and CRP concentrations. Walnuts were provided raw, shelled, and in individualized portions calculated to provide 12% of daily energy intake. Subjects were randomly assigned to two treatment sequences: either to adhere to their habitual diet for six months followed by their habitual diet plus walnuts for the next six months; or, to consume their habitual diet plus walnuts for six months followed by a six month period of their habitual diet. Subjects were not asked to make any changes in their usual diet, medication or dietary supplement use.
Walnuts are a concentrated source of \( \gamma \)-tocopherol and although the unadjusted levels of \( \gamma \)-tocopherol were not different on the walnut diet, walnut consumption resulted in lower plasma lipids and a significantly higher ratio of \( \gamma \)-tocopherol to cholesterol.

Tocopherols in blood are associated with lipoproteins and the molar ratio of tocopherol to cholesterol better reflects status (20). Unlike \( \alpha \)-tocopherol, \( \gamma \)-tocopherol is not maintained in the blood and large increases in intake are reflected in relatively small changes in plasma levels. It is now known that \( \alpha \)-tocopherol and \( \gamma \)-tocopherol are equally well absorbed and secreted by the intestine into chylomicron lipoprotein (21-22). However, \( \alpha \)-tocopherol is preferentially secreted by the liver into nascent very-low-density lipoprotein via a cytosolic liver tocopherol-binding protein. (23). RRR-\( \alpha \)-tocopherol has a greater affinity for this tocopherol-binding protein than RRR-\( \gamma \)-tocopherol. The tocopherol binding protein is the major determinant of plasma tocopherol levels. The concentration of \( \gamma \)-tocopherol in tissues such as coronary endothelial cells may be higher than that of plasma as tissues receive \( \gamma \)-tocopherol from chylomicrons (24). Walnuts contain practically no \( \alpha \)-tocopherol and blood concentration of \( \alpha \)-tocopherol were lower on the walnut diet. However, the ratios of \( \alpha \)-tocopherol to cholesterol did not differ between the diets which indicates that nutrient status with respect to \( \alpha \)-tocopherol was not compromised on the walnut diet.

Vitamin E supplement users exhibited higher levels of \( \alpha \)- and lower levels of \( \gamma \)-tocopherol than nonusers both on the habitual and on the walnut diet. This has been observed in other studies (16, 24-25). It is likely that supplementation with \( \alpha \)-tocopherol
displaces γ-tocopherol from the hepatic tocopherol-binding protein thus depressing the levels of γ-tocopherol in plasma.

There has been an increasing interest in the role of γ-tocopherol in disease prevention (26-27). Both α- and γ-tocopherol are potent lipophilic antioxidants and γ-tocopherol uniquely scavenges reactive nitrogen species (15). Gamma-tocopherol is more potent than α-tocopherol with respect to inhibition of cyclooxygenase in cell systems and in decreasing proinflammatory eicosanoids and inflammation damage in rats (17). Also, γ-tocopherol was shown to significantly attenuate oxidized LDL mediated activation of transcription factor NF-κB pathway and apoptosis in human coronary artery endothelial cells (28).

The clinical implications of blood γ-tocopherol levels are not clear. Some studies show that decreases in plasma γ-tocopherol are associated with atherosclerosis and heart disease (29-30) whereas others do not support a protective effect (27).

In addition to being a source of γ-tocopherol, walnuts are also one of the richest plant sources of the n-3 fatty acid alpha-linolenic acid. There is some evidence showing that n-3 fatty acids may have anti-inflammatory effects. Supplementation with n-3 fatty acids has been reported to inhibit mononuclear cell proliferation and monocyte cytokine production (13). The question we attempted to answer was whether walnut consumption would have an anti-inflammatory effect as reflected in blood levels of CRP. Recently, CRP has been identified as a marker of systemic inflammation and associated with increased risk of cardiovascular disease, myocardial infarction and stroke (7).
Walnut consumption did not influence CRP levels among non users of vitamin E supplement. However, the walnut diet resulted in significantly higher CRP levels in vitamin E supplement users. The determinants of CRP levels in our subjects were consistent with those found by other researchers. CRP levels were positively associated with BMI and negatively associated with NSAIDs use and vitamin E supplement use.

To determine whether the observed increase in CRP levels on the walnut diet may be related to alterations in plasma α- and γ-tocopherol, the association between plasma CRP levels and the ratios of α- and γ-tocopherol to cholesterol was examined. As shown in Figures 3 and 4, CRP levels were higher on the walnut diet but only in those with a high ratio of α- and a low ratio γ-tocopherol to cholesterol respectively. Regular use of vitamin E supplements is expected to increase the ratio of α-tocopherol to cholesterol and to decrease the ratios of γ-tocopherol to cholesterol. Why CRP levels were higher on the walnut diet in vitamin E supplement users and in those with higher blood levels of α-tocopherol and lower blood levels of γ-tocopherol is not clear.

Multiple regression models with CRP as the outcome variable show that both dietary α-tocopherol intake from food plus supplements and dietary γ-tocopherol intake are negatively associated with plasma CRP on both the habitual diet and the walnut diet. This indicates that dietary intake of both α- and γ- tocopherol modulate CRP levels.
References


15. Chisten S. Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. γ-Tocopherol traps mutagenic electrophiles such as NOx and


TABLE 1. Characteristics of the Participants at Baseline

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td>(n = 40)</td>
<td>(n = 50)</td>
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<tr>
<td>Age (y)</td>
<td>53.13±11.77</td>
<td>55.38±9.65</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.49±2.59</td>
<td>25.36±3.93</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>221.21±36.01</td>
<td>227.75±33.42</td>
</tr>
<tr>
<td>Vitamin E supplement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Non-users</td>
<td>33</td>
<td>41</td>
</tr>
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</table>

¹Mean ± SD
TABLE 2. Serum Cholesterol, α-, γ- Tocopherols, Ratio of α-, γ- Tocopherols to Cholesterol and C-reactive Protein for the Habitual and Walnut Supplemented Diet

<table>
<thead>
<tr>
<th></th>
<th>Habitual diet</th>
<th>Walnut diet</th>
<th>Difference walnut minus habitual diet</th>
<th>Interaction</th>
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<tr>
<td></td>
<td>LS Mean 1</td>
<td>95% CI 2</td>
<td>LS Mean</td>
<td>95% CI</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>All subjects*</td>
<td>5.51</td>
<td>5.29-5.72</td>
<td>5.36</td>
<td>5.16-5.57</td>
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<tr>
<td>Vitamin E supplement non user</td>
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<td>5.21-5.71</td>
<td>5.36</td>
<td>5.13-5.61</td>
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<td>5.34-6.18</td>
<td>5.34</td>
<td>4.96-5.75</td>
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<td>Alpha-tocopherol (μmole/L)</td>
<td></td>
<td></td>
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<tr>
<td>All subjects*</td>
<td>33.761</td>
<td>30.973-36.803</td>
<td>32.308</td>
<td>29.64-35.219</td>
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<td>39.211-56.664</td>
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<td>35.442-51.17</td>
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<td>Alpha-tocopherol:cholesterol (μmole/mm mole)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>All subjects*</td>
<td>6.129</td>
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<td>2.39</td>
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<td>2.345-3.317</td>
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<td>1.344</td>
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<td>Gamma-tocopherol:cholesterol (μmole/mm mole)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects*</td>
<td>0.428</td>
<td>0.360-0.510</td>
<td>0.459</td>
<td>0.386-0.547</td>
</tr>
<tr>
<td>Vitamin E supplement non user*</td>
<td>0.512</td>
<td>0.427-0.616</td>
<td>0.538</td>
<td>0.448-0.646</td>
</tr>
<tr>
<td>Vitamin E supplement user*</td>
<td>0.213</td>
<td>0.147-0.309</td>
<td>0.254</td>
<td>0.175-0.368</td>
</tr>
<tr>
<td>C-reactive protein (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects*</td>
<td>1585.6</td>
<td>1293.7-1943.3</td>
<td>1759.5</td>
<td>1436.5-2155.2</td>
</tr>
<tr>
<td>Vitamin E supplement non user*</td>
<td>2142.7</td>
<td>1731.5-2651.4</td>
<td>2150.0</td>
<td>1737.4-2660.5</td>
</tr>
<tr>
<td>Vitamin E supplement user*</td>
<td>1173.3</td>
<td>828.6-1661.5</td>
<td>1439.9</td>
<td>1018.2-2036.2</td>
</tr>
</tbody>
</table>

1 Least Square Mean  
2 95% Confidence Interval  
* Statistically significant different from habitual diet, p < 0.05
TABLE 3. CRP (ng/ml) Concentration of Participants by Gender, Usage of NSAIDs, Usage of Vitamin E Supplement and Usage of Lipid Lowering Medications\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>LS Mean(^2)</th>
<th>95% CI(^2)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>47</td>
<td>2065</td>
<td>1599</td>
<td>2668</td>
</tr>
<tr>
<td>Males</td>
<td>32</td>
<td>1567</td>
<td>1149</td>
<td>2137</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>+31.78%</td>
<td>-11.85%</td>
<td>+96.99%</td>
</tr>
<tr>
<td><strong>NSAIDs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users</td>
<td>21</td>
<td>1129</td>
<td>693</td>
<td>1839</td>
</tr>
<tr>
<td>Non-users</td>
<td>58</td>
<td>2206</td>
<td>1816</td>
<td>2682</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>-48.83%</td>
<td>-69.59%</td>
<td>-13.96</td>
</tr>
<tr>
<td><strong>Vitamin E supplement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users</td>
<td>21</td>
<td>1193</td>
<td>824</td>
<td>1726</td>
</tr>
<tr>
<td>Non-users</td>
<td>58</td>
<td>2165</td>
<td>1732</td>
<td>2703</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>-44.89%</td>
<td>-64.21%</td>
<td>-15.16%</td>
</tr>
<tr>
<td><strong>Lipid lowering medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users</td>
<td>3</td>
<td>1013</td>
<td>366</td>
<td>2803</td>
</tr>
<tr>
<td>Non-users</td>
<td>76</td>
<td>1891</td>
<td>1545</td>
<td>2314</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>-46.42%</td>
<td>-81.02%</td>
<td>+51.19%</td>
</tr>
</tbody>
</table>

\(^1\) CRP concentration are of those obtained on the 4\(^{th}\) and 6\(^{th}\) month of the habitual diet

\(^2\) values are least-squares means and 95% confidence intervals
TABLE 4. Multiple Linear Regression Results with CRP Levels Set As the Dependent Variable and Dietary $\alpha$-Tocopherol, Dietary $\gamma$-Tocopherol, BMI and NSAIDs Set As Independent Variables

<table>
<thead>
<tr>
<th>Diet and model variables</th>
<th>Model</th>
<th>Standardized $\beta$</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td></td>
<td></td>
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<tr>
<td>Habitual Diet</td>
<td>0.27</td>
<td>-</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Dietary $\alpha$-tocopherol</td>
<td></td>
<td>-0.29</td>
<td>0.005</td>
</tr>
<tr>
<td>Dietary $\gamma$-tocopherol</td>
<td></td>
<td>-0.24</td>
<td>0.019</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td>0.28</td>
<td>0.008</td>
</tr>
<tr>
<td>NSAIDs</td>
<td></td>
<td>-0.22</td>
<td>0.030</td>
</tr>
<tr>
<td>Walnuts Supplemented Diet</td>
<td>0.24</td>
<td>-</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Dietary $\alpha$-tocopherol</td>
<td></td>
<td>-0.16</td>
<td>0.104</td>
</tr>
<tr>
<td>Dietary $\gamma$-tocopherol</td>
<td></td>
<td>-0.26</td>
<td>0.015</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td>0.28</td>
<td>0.009</td>
</tr>
<tr>
<td>NSAIDs</td>
<td></td>
<td>-0.27</td>
<td>0.010</td>
</tr>
</tbody>
</table>
Note:
Habitual diet: usual diet
Walnuts diet: usual diet + walnuts 12% of energy intake

FIGURE 1. The study design
FIGURE 2. The relation between baseline BMI (kg/m²) and log (CRP) in participants ($\beta = 0.088 \pm 0.029$, $p = 0.002$).
* statistically significant different from habitual diet, p < 0.05

1 ratio of α-tocopherol to cholesterol higher than median

2 ratio of α-tocopherol to cholesterol lower than median

FIGURE 3. Least-squares means and 95% confidence intervals of CRP concentrations (ng/ml) in all participants with a high\(^1\) and low\(^2\) molar ratio of α-tocopherol to cholesterol on the habitual and walnuts supplemented diet
Habitual Diet □ Walnuts Supplemented Diet

* statistically significant different from habitual diet, p < 0.05

1 ratio of α-tocopherol to cholesterol higher than median

2 ratio of α-tocopherol to cholesterol lower than median

FIGURE 4. Least-squares means and 95% confidence intervals of CRP concentrations (ng/ml) in all participants with a high\(^1\) and low\(^2\) molar ratio of \(γ\)-tocopherol to cholesterol on the habitual and walnuts supplemented diet
CHAPTER 5
INFLAMMATORY MEDIATORS PUBLISHABLE PAPER

The Effects of Long Term Walnuts Consumption on Inflammatory Mediators in Healthy Individuals

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**Introduction**

The concept that food components offer benefits beyond basic nutrition is relatively new to the scientific community. This knowledge continues to be supported by research, which demonstrates that a health promoting diet is defined as much by foods consumed as by those avoided.

The favorable metabolic outcomes of consuming walnuts is relatively novel and thought to be due to a lipid composition low in saturated fatty acids (SFA) and high in polyunsaturated fatty acids (PUFA). Although the nutrient contribution of walnuts is similar to that of other tree nuts, it is distinguished by a high proportion of the n-3 fatty acids and α-linolenic acid (ALA, 18:3, n-3).

According to prospective cohort studies such as the Adventist Health Study, the Nurses Health Study, the Iowa Woman’s Health Study and the Physician’s Health Study more frequent nut consumption is correlated with cardiovascular risk reduction and is inversely associated with all cause mortality (1-4). In addition, clinical human intervention studies which involve adding walnuts to the diet are consistent in showing decreases in total cholesterol and low density lipoprotein (LDL)-cholesterol (5-9).

Studies have shown that long term supplementation of capsules from fish oil containing the long chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduced production of proinflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) in healthy volunteers (10-13). The inhibition of IL-1, IL-6 and TNF production is accompanied by a decreased ratio of arachidonic acid (AA) to EPA in the membrane phospholipids of mononuclear cells (10). EPA can be formed endogenously from the 18-carbon fatty acid,
α-linolenic acid, and foods containing n-3 fatty acids such as flaxseed oil and fish oil have received more attention in nutrition research.

Nonetheless, the effect of walnuts, known as rich source of ALA, on proinflammatory mediator synthesis has not yet been examined. There is as yet no information regarding the association between walnuts ingestion, antioxidant status and proinflammatory cytokines production. Therefore, the purpose of the present study is to determine whether the addition of walnuts to the diet produces similar effects on reduction of proinflammatory mediators and cytokines as those observed in fish and flaxseed oil.

**Subjects and Methods**

**Subjects**

Subjects for this study were selected from participants in the walnut body weight trial conducted by Joan Sabaté and colleagues at the Department of Nutrition, School of Public Health, Loma Linda University.

Eligibility criteria were no significant weight changes during previous six months, not over or under weight, non-diabetic, and no known allergy to nuts. Subjects were also requested to have their habitual diet closely related to the typical American diet with nuts consumption less than once per week. Subjects were also required to maintain their activities, exercise and other lifestyle habits throughout the study. Any signs of illness experienced and, or supplements or medications ingested were recorded.

Thirty participants met eligibility criteria for the cytokines sub-study. Selected subjects consumed no multivitamin, vitamin E, flax seed, fish oil supplements, no anti-inflammatory medications taken, no history of heart diseases and inflammatory related
diseases reported, and established at least 10% of energy intake from walnuts during the walnut supplemented diet phase as reflected in the 7 of 24 hour dietary recalls.

The Institutional Review Board of Loma Linda University approved the study. Each subject received an explanation and signed an informed consent before being included in the study.

Study Design

This was a 12-month randomized crossover intervention study, which consisted of two phases, walnut supplemented diet phase and habitual diet phase. Subjects were randomly assigned to either of these two phases for the first six months and then switched to the other phase for the second six months. During the walnut supplemented diet phase, subjects volunteered to incorporate a portion of walnuts equal to 12% of their daily energy intake into their habitual diet. Subjects followed their habitual diet for habitual diet phase. No other dietary advice was given to both phases. Figure 1

Prepackages daily allowances of raw, shelled walnuts were provided in amounts varying from 28 to 56g, according to subjects’ total energy intake. No instructions as to time of the day or cooking method were concerned.

The composition of walnuts was reviewed according to the United State Department of Agricultural (USDA). One hundred gram of edible portion of walnuts contain 6.126 g of total saturated fatty acids, 8.933 g of total monounsaturated fatty acids (8.799 g of 18:1 and 0.134 of 20:1 isomers), 47.174 g of total polyunsaturated fatty acids (38.093 g of 18:2, 9.080 g of 18:3 isomers).
Blood specimens were collected at the end of month 4, 6, 10, and 12 for future laboratory analyses. Compliance to the prescribed diet was ensured and assessed by 7 random 24-hour recall during each diet period.

**Laboratory Analyses**

After the subjects had fasted overnight, blood was collected by venipuncture into evacuated tubes that were contained heparin as anticoagulant. Blood specimens then were centrifuged at 2.7 x g for 10 min at 4 °C. The plasma was portioned and stored at -80 °C for future laboratory analyses at nutrition department, School of Public Health Loma Linda University.

The quantitative determination of human soluble E-Selectin, IL-1β, IL-6 and TNF-α were measured by an ultrasensitive ELISA (R&D Systems, Minneapolis).

**Statistical Analyses**

The effect of walnuts on proinflammatory cytokines including E-Selectin, IL-1β, IL-6, and TNF-α was examined by mixed-effect models, with fixed-effect terms for diet and period. Although the data was correlated, the correlations did not markedly decline over time, and therefore the covariance structures were modeled using compound symmetry model. In addition, gender-diet interactions were tested by adding appropriate fixed terms to the model. Correlation analysis was employed to check for possible relations between each of the proinflammatory cytokines and serum tocopherols. All outcome and tocopherol values were normalized by log transformation prior to analysis. However, for ease of interpretation, final results were presented in original units.
Results

Blood specimens from thirty participants were analyzed for inflammatory cytokines levels. No adverse effects due to the consumption of walnuts were observed or reported. Participant’s baseline characteristics including age and BMI are shown in Table 1.

Least square means and 95% confidence intervals of e-selectin, TNF-α, IL-1β, and IL-6 during habitual and walnuts supplemented diet are shown in Table 2. There were no significant differences of the plasma e-selectin and TNF-α in the group as a whole. Among female participants, e-selectin and TNF-α increased 3.91% and 5.74% respectively on the walnut supplemented diet. In contrast, both inflammatory markers decreased but not significantly among male participants on the walnut supplemented diet.

Plasma levels IL-1β and IL-6 were lower on the walnuts supplemented diet in the combined group of male and female participants (p= 0.0015, p= 0.017, respectively). However, in contrast with the other two inflammatory markers, plasma levels of IL-1β and IL-6 significantly dropped only in the male group of participants Table 2.

Discussion

The anti-inflammatory effects of n-3 fatty acid have been extensively studied. However, the effect of n-3 fatty acid on inflammation, atherosclerosis and the risk of heart disease is somewhat controversial. In a healthy male population, Ascherio et al., (14) did not find a substantial reduction on the risk of coronary heart disease among healthy men after increasing fish intake from one to two servings to five to six serving per week. Furthermore, in the Physicians’ Health Study there was no evidence for an association between dietary intake of fish and any cardiovascular endpoint (15).
Moderate fish intake was not associated with a reduced risk of cardiovascular disease. In contrast, Mori et al., found that supplementation of 3.65 g/d of n-3 fatty acids for 12 weeks significantly reduced platelet aggregation whereas, the GISSI-Prevenzione trial suggested that 1 g/d of n-3 fatty acids reduced the risk of cardiovascular death in myocardial infarction patients (16-17).

Regarding the anti-inflammatory effects of n-3 fatty acids, diets supplemented with n-3 or n-6 fatty acids, or a combination of both exerted different effects on inflammatory response, which is probably related to differing roles on the modulation of inflammatory response. Supplementation of 4.5 g per day of n-3 fatty acids for 6 weeks has been shown to suppress IL-1β, IL-1α, IL-2 and TNF in healthy adults (18-19). In contrast, Chan et al., documented the lack of effect of fish oil fed at a level of 4 g per day on CRP, TNF and IL-6 in obese individuals after 6 weeks treatment (20).

In this study, we examined the relation between the consumption of walnuts rich in α-linolenic acid and plasma concentrations of biomarkers of inflammation among healthy participants. Compared to habitual diet, there was no effect of walnuts consumption on plasma concentrations of e-selectin and TNF-α. However, we found an inverse relationship between walnuts consumption and plasma concentrations of IL-1β and IL-6. IL-1β levels were 13.4% lower whereas, IL-6 levels were 16.06% lower during walnuts supplemented diet. These associations were independent of lifestyle and physiologically different since it was crossover design trial.

The relevance of inflammation in the atherogenic process was suggested recently by several studies. For years, the main cause of atherosclerosis was thought to be the accumulation of lipids in the arterial wall. However, there is growing evidence which
suggests that markers of inflammation are strong predictors of cardiovascular disease (21). These markers include the proinflammatory cytokines such as IL-6, TNF-α and IL-1β and adhesion molecules such as e-selectin (21-25). It is suggested that oxidized low density lipoprotein triggers a proinflammatory response and that IL-6 may have a major role in the mediation of the inflammatory and immune responses initiated by oxidation and injury (22-23). In addition, an association between proinflammatory cytokines obesity and coronary heart disease has been demonstrated (24). Moreover, e-selectin, a soluble cell adhesion molecule, increases after inflammation, and elevated e-selectin levels are detected among coronary artery disease patients (25).

One plausible mechanism by which n-3 fatty acids modulate inflammation is by lowering hydrogen peroxide production. Hydrogen peroxide is a major activator of the nuclear factor-κB system, the coordinator of expression of adhesion molecules upon cytokine stimulation. Multiple double bonds in the carbon structure of n-3 fatty acids react first with active oxygen species thus sparing other molecules. This effect produces a reduction in the amounts of hydrogen peroxide in the system (26-27).

In vitro studies demonstrated that the inhibitory effects of polyunsaturated fatty acids on inflammation are different depending on the number of unsaturated double bonds of n-3 fats. The ability to inhibit inflammation increases with the number of unsaturations, and the carbon chain length is not a factor (26, 28). Thus, the most potent inhibitor of the inflammation activation in vitro is the docosahexaenoic acids. However, there is no confirmation about this plausible mechanism in any of in vivo study. Our finding suggested that walnuts, rich in polyunsaturated fatty acids, had similar inverse associations with some of the biomarker of inflammation, especially IL-1β and IL-6.
The human body is capable of converting a portion of \(\alpha\)-linolenic acid to EPA or DHA. This conversion is inefficient and depends on the quantity of n-6 and n-9 fatty acids intake since these fatty acids compete with n-3 fatty acids for desaturase enzyme in the synthetic pathway. Thus, the EPA and DHA, highly unsaturated fatty acids, are 2.5-5 times more effective in modulating inflammatory cytokines than \(\alpha\)-linolenic acid. This might explain the results of our findings of no alteration in plasma levels of TNF-\(\alpha\) and e-selectin. Moreover, the high content of the n-6 fatty acids, linoleic acids, in walnuts may have inhibited the full effect of \(\alpha\)-linolenic acid.

In conclusion, this study suggests that walnut consumption inversely associated with inflammatory factors including IL-1\(\beta\) and IL-6, which may explain in part the beneficial effect of nut consumption in lowering the risk of heart disease.
References


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<tr>
<th></th>
<th>Male</th>
<th>Female</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>43±9.37</td>
<td>52.33±10.18</td>
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<tr>
<td>BMI</td>
<td>27.59±3.41</td>
<td>25.95±4.01</td>
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#TABLE 2
Least-squares Means and 95% Confidence Intervals of E-selectin, TNF-α, IL-1β, and IL-6 During Habitual and Walnut Supplemented Diet

<table>
<thead>
<tr>
<th></th>
<th>Habitual diet</th>
<th>Walnuts supplemented diet</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS Mean¹</td>
<td>95% CI</td>
<td>LS Mean</td>
</tr>
<tr>
<td>e-selectin (ng/mL)</td>
<td>42.9</td>
<td>35.56</td>
<td>51.77</td>
</tr>
<tr>
<td></td>
<td>37.51</td>
<td>29.85</td>
<td>47.14</td>
</tr>
<tr>
<td></td>
<td>49.07</td>
<td>36.08</td>
<td>66.73</td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
<td>1.107</td>
<td>0.988</td>
<td>1.241</td>
</tr>
<tr>
<td></td>
<td>1.15</td>
<td>1.001</td>
<td>1.321</td>
</tr>
<tr>
<td></td>
<td>1.066</td>
<td>0.884</td>
<td>1.284</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
<td>1.841</td>
<td>1.382</td>
<td>2.451</td>
</tr>
<tr>
<td></td>
<td>1.938</td>
<td>1.369</td>
<td>2.746</td>
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<td></td>
<td>1.748</td>
<td>1.094</td>
<td>2.793</td>
</tr>
<tr>
<td>IL-1β (ng/mL)</td>
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<td>0.29</td>
<td>0.383</td>
</tr>
<tr>
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<td>0.353</td>
<td>0.294</td>
<td>0.425</td>
</tr>
<tr>
<td></td>
<td>0.314</td>
<td>0.251</td>
<td>0.394</td>
</tr>
</tbody>
</table>

¹Least Squares Mean and 95% Confidence Intervals

TNF-α, tumor necrosis factor-α
IL-1β, interleukin-1β
IL-6, interleukin-6
Note:
Habitual diet: usual diet
Walnuts diet: habitual diet + walnuts 12% of energy intake

FIGURE 1. The study design
CHAPTER 6

CONCLUSION

A. Summary and Implications of Findings

In this 12 month feeding trial study, we examined the relationship between long term walnut consumption, rich in n-3 fatty acids and γ-tocopherol, and plasma concentrations of inflammatory mediators including IL-1β, IL-6, e-selectin, TNF-α, and CRP in healthy men and women participants.

Walnuts are an excellent source of γ-tocopherol, however we found that increasing the amount of walnuts in a diet insignificantly increased plasma γ-tocopherol concentration. In addition, α-tocopherol level was significantly decreased as walnuts supplemented for 12% of daily energy intake. The addition of walnuts of 28-56 g per day into diet may have decreased the level of α-tocopherol concentration. The mechanism by which walnuts, rich in γ-tocopherol, decreased the plasma concentration of α-tocopherol is unclear and need further investigation. One of the proposed mechanisms is the competitive absorption rate of both tocopherols.

The fatty acid profiles of many nuts have been found to have beneficial effects. Walnuts have a unique profile that is high in n-3 fatty acids, which have been shown to have positive outcomes on the inflammatory related disease such as heart disease, and cancer. The effects of long term walnuts consumption on the inflammatory mediators were interesting. The findings proved that this amount of walnuts consumption may be a benefit in inflammatory related diseases.
B. Limitations

This study was an intervention study, thus was subject to difficulties in controlling walnut consumption. Participants’ compliance was monitored. However, given the length and scope of the trial it was often difficult for participants to comply 100% with the protocol. Moreover, multiple clinic visits were burdensome to participants to complete. Several follow-up clinics were set up to minimize this limitation.

The amounts of the specimen were also one of the important limitations. Since the analyses were conducted after the study had been completed, utilizing specimen for all laboratory analysis was a concern.

C. Conclusions and Future Research

Incorporating walnuts as a regular part of the diet may be of concern in those who take vitamin E supplement because of the interaction. The beneficial effects of vitamin E supplement may be compromised by dietary walnuts intake. However, the biological mechanism needs further investigation.

Comparison of n-3 fatty acids from walnuts and other high n-3 fatty acids foods such as fish oil on the inflammatory effect would be an interesting study for the future.
REFERENCES


Kasparek S. Chemistry of tocopherols and tocotrienols. New York: Marcel Dekker, 1980


