The Potential Effect of Alpha-Linolenic Acid on Prostate Specific Antigen

by

Jiali Xu

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

© Copyright by Jiali Xu (2016)
The Potential Effect of Alpha-Linolenic Acid on Prostate Specific Antigen

Jiali Xu

Master of Science

Graduate Department of Nutritional Science
University of Toronto

2016

Abstract

The objectives were to investigate the effect of alpha-linolenic acid (ALA) on prostate specific antigen (PSA) through two studies: 1) Determine the effect of ALA-enriched diet on PSA levels and serum ALA concentrations, through a secondary analysis of a randomized controlled trial in type 2 diabetes patients. 2) Determine the effect of ALA on prostate cancer cell growth and PSA production. Results: 1) ALA supplementation resulted in a reduction of 0.43 ng/ml on PSA (P=0.014). 2) ALA-enriched diet increased percentage composition of ALA in total lipids, phospholipids, free fatty acids and triglycerides (P<0.05). 3) No inhibitory effect of ALA was found in cell growth and PSA production by prostate cancer cells when provided at physiological doses. Conclusions: Although serum PSA levels were lowered after ALA supplementation. The in vitro study detected no inhibitory effect on cell growth and PSA production. More studies are needed to fill in the gap.

Word Count: 149
# Table of Contents

Table of Contents........................................................................................................................................iii
List of Abbreviations.....................................................................................................................................viii
List of Tables...............................................................................................................................................x
List of Figures.............................................................................................................................................xi
List of Appendix ........................................................................................................................................xiv
Chapter 1 Introduction ................................................................................................................................1
  1 Introduction...........................................................................................................................................1
Chapter 2 Literature Review ..........................................................................................................................4
  2 Literature Review ....................................................................................................................................4
    2.1 Epidemiology of Prostate Cancer .......................................................................................................4
    2.2 The Role of Dietary fat on Prostate Cancer .........................................................................................4
      2.2.1 Total Fat ......................................................................................................................................5
      2.2.2 Saturated Fat ..............................................................................................................................5
      2.2.3 Monounsaturated Fat ................................................................................................................5
        2.2.3.1 Omega-6 Polyunsaturated Fat ...............................................................................................6
          2.2.3.1.1 Linoleic acid ......................................................................................................................6
          2.2.3.1.2 Arachidonic acid .............................................................................................................7
        2.2.3.2 Omega-3 Polyunsaturated Fat ...............................................................................................7
          2.2.3.2.1 EPA and DHA ...................................................................................................................8
          2.2.3.2.2 Alpha-Linolenic Acid .......................................................................................................9
            2.2.3.2.2.1 Meta-Analysis ..........................................................................................10
            2.2.3.2.2.2 Prospective Studies ..........................................................................................11
              2.2.3.2.2.2.1 Dietary ALA Intake in Prospective Studies ......11
              2.2.3.2.2.2.2 Blood ALA Levels in Prospective Studies......12
            2.2.3.2.2.3 Case-Control Studies ............................................................................................13
              2.2.3.2.2.3.1 Dietary ALA Intake in Case-Control Studies .....13
              2.2.3.2.2.3.2 Blood ALA Levels in Case-Control Studies ......13
            2.2.3.2.2.4 Intervention Studies ............................................................................................14
            2.2.3.2.2.5 Cell Culture Studies ............................................................................................15
2.2.3.2.6 Animal Studies................................................................. 15

2.3 Possible Mechanisms........................................................................................................... 16

2.3.1 Eicosanoid synthesis ........................................................................................................ 16

2.3.2 Free radical formation....................................................................................................... 18

2.4 Prostate specific antigen as a biomarker of prostate cancer............................................. 18

2.5 Effect of Type 2 Diabetes on Prostate Cancer ................................................................. 20

Chapter 3 Hypothesis and Objectives..................................................................................... 24

3 Hypothesis and Objectives .................................................................................................. 24

3.1 Hypothesis.......................................................................................................................... 24

3.2 Objectives.......................................................................................................................... 24

Chapter 4 Secondary Analysis from a Randomized Control Trial: The Effect of
Alpha-Linolenic Acid Supplementation on Serum Prostate Specific Antigen......................... 25

4 Secondary Analysis from a Randomized Control Trial: The Effect of Alpha-Linolenic Acid
Supplementation on Serum Prostate Specific Antigen ............................................................. 25

4.1 Abstract.................................................................................................................................. 25

4.2 Introduction............................................................................................................................ 26

4.3 Methods................................................................................................................................ 26

4.3.1 Study Participants and Protocol for Original Study......................................................... 26

4.3.2 Dietary Intervention.......................................................................................................... 27

4.3.3 Study Population and Protocol for the Current Study..................................................... 27

4.3.4 PSA measurement............................................................................................................. 28

4.3.5 Fatty Acids Analysis......................................................................................................... 28

4.3.5.1 Internal Standard Preparation ...................................................................................... 28

4.3.5.2 Extraction of lipids........................................................................................................ 28

4.3.5.3 Separation of Lipid Fractions ....................................................................................... 29

4.3.5.4 Preparation of Fatty Acid Methyl Esters (FAMEs)...................................................... 29

4.3.5.5 Gas Chromatography .................................................................................................. 29

4.3.5.6 Calculation..................................................................................................................... 30

4.3.5.6.1 The Concentrations of Fatty Acids........................................................................... 30

4.3.5.6.2 The Percentage Composition of Fatty Acids.......................................................... 30

4.3.6 Statistical Analysis............................................................................................................. 30
Chapter 5 The Effect of ALA on Human Prostate Cancer Cells

5.1 Abstract .................................................................................................................. 47
5.2 Introduction .......................................................................................................... 48
5.3 Methods ................................................................................................................. 49
  5.3.1 Cell Line ............................................................................................................ 49
  5.3.2 Subculture procedure ....................................................................................... 49
  5.3.3 Cell Culture Plating and Treatment ............................................................... 49
Chapter 5 Results

5.3.4 Treatment medium ...........................................................................................................50
5.3.5 PSA Measurement ............................................................................................................50
5.3.6 Cell Proliferation Assay ................................................................................................51
5.3.7 Statistical Analysis ..........................................................................................................51
5.4 Results ................................................................................................................................52
5.4.1 Descriptive Data from Clinical Trial ..............................................................................52
5.4.2 Cell Proliferation Assay (MTS) .......................................................................................52
  5.4.2.1 Alpha-Linolenic Acid ..................................................................................................52
  5.4.2.2 Linoleic Acid ..............................................................................................................52
  5.4.2.3 Oleic Acid ..................................................................................................................52
  5.4.2.4 Stearic Acid ..............................................................................................................52
  5.4.2.5 C18 Fatty Acids .........................................................................................................53
5.4.3 PSA Produced from the Cells ..........................................................................................53
  5.4.3.1 Alpha-Linolenic Acid ................................................................................................53
  5.4.3.2 Linoleic Acid .............................................................................................................53
  5.4.3.3 Oleic Acid ................................................................................................................53
  5.4.3.4 Stearic Acid ..............................................................................................................54
  5.4.3.5 C18 Fatty Acids .........................................................................................................54
5.4.4 Correlation of C18 fatty acids and Cell growth ...............................................................54
5.4.5 Correlation of C18 fatty acids and PSA ........................................................................54
5.4.6 Correlation of Cell growth and PSA .............................................................................54
5.5 Discussion ...........................................................................................................................55
5.6 Strengths and Limitations ..................................................................................................57
5.7 Conclusion .........................................................................................................................57

Chapter 6 Overall Discussion ..................................................................................................68
6 Overall Discussion .................................................................................................................68
  6.1 Discussion .........................................................................................................................68
  6.2 Future Work .......................................................................................................................69

Chapter 7 Conclusion ...............................................................................................................71
7 Conclusion .............................................................................................................................71
References ....................................................................................................................................72
Appendices.................................................................................................................................81
List of Abbreviations

ADT - Androgen Deprivation Therapy
AR - Androgen Receptor
ARA - Arachidonic Acid
CHD - Coronary Heart Disease
CI - Confidence Interval
COX - Cyclooxygenase
CV - Coefficient of Variation
DHA - Docosahexaenoic Acid
DHT - 5α-dihydrotestosterone
DMAB - 3,2′-dimethyl1,4-aminobiphenyl
EPA - Eicosapentaenoic Acid
FASN - Fatty Acid Synthase
FBS - Fetal Bovine Serum
FFQ - Food Frequency Questionnaire
GLA - Gamma Linoleic Acid
HETE - Hydroxyeicosatetraenoic Acid
LA - Linoleic Acid
LOX - Lipoxygenase
LT - Leukotriene
LTS - Lipoteichoic Acid
NSAID - Non-Steroidal Anti-Inflammatory Drug
MTS - 3-(4,5-dimethylthiazol-2-yl)-
5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt;
PG - Prostaglandin
PHS - Pooled Human Serum
PUFA - Polyunsaturated Fatty Acid
OR - Odds Ratio
PSA - Prostate specific antigen
RPMS - Roswell Park Memorial Institute
RR - Relative Risk
SD - Standard Deviation
SE - Standard Error
SREBP-1 - Sterol Response Element Binding Protein-1
TLC - Thin Layer Chromatography
TP - Testosterone Propionate
\( \mu \text{M} - \mu\text{mol/L} \)
List of Tables

Chapter 4

Table 1 - Nutrition facts of study bread (per 500kcal)

Table 2 - Characteristics of study subjects at baseline

Chapter 5

Table 3 - Descriptive data of fatty acids in serum samples (μM)
List of Figures

Chapter 4

Figure 1 - Metabolism of n-6 and n-3 fatty acids

Figure 2 - Synthesis of eicosanoids through COX pathway

Figure 3 - Metabolism of AA and EPA through LOX pathway

Figure 4 - Flow of participants through the study

Figure 5 - Effect of Diet on PSA

Figure 6a - Percentage composition of total lipid ALA during the study

Figure 6b - Percentage composition of phospholipid ALA during the study

Figure 6c - Percentage composition of triglyceride ALA during the study

Figure 6d - Percentage composition of FFA ALA during the study

Figure 7a - Total lipid ALA concentrations during the study

Figure 7b - Phospholipid ALA concentrations during the study

Figure 7c - Triglyceride ALA concentrations during the study

Figure 7d - FFA ALA concentrations during the study

Figure 8a - Correlation of change in PSA and change in total lipid ALA (percentage composition) during the study (with outliers)

Figure 8b - Correlation of change in PSA and change in FFA ALA (percentage composition) during the study (with outliers)
Figure 9 - Correlation of change in PSA and change in triglyceride ALA (concentrations) during the study (with outliers)

Chapter 5

Figure 10a - Effect of different ALA concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 10b - Effect of different LA concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 10c - Effect of different oleic acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 10d - Effect of different stearic acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 10e - Effect of different C18:0 fatty acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 11a - Effect of different ALA concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 11b - Effect of different LA concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
Figure 11c - Effect of different oleic acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 11d - Effect of different stearic acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 11e - Effect of different C18:0 fatty acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 12a - Correlation of LNCaP cell growth and ALA concentrations

Figure 12b - Correlation of LNCaP cell growth and LA concentrations

Figure 12c - Correlation of LNCaP cell growth and oleic acid concentrations

Figure 12d - Correlation of LNCaP cell growth and stearic acid concentrations

Figure 13a - Correlation of PSA produced from the cells and ALA acid concentrations

Figure 13b - Correlation of PSA produced from the cells and ALA acid concentrations

Figure 13c - Correlation of PSA produced from the cells and ALA acid concentrations

Figure 13d - Correlation of PSA produced from the cells and ALA acid concentrations

Figure 14 - Correlation of LNCaP cell growth and PSA produced from the cells
List of Appendix

Appendix 1 - Effect of Diet on Percentage Composition of Linoleic Acid

Appendix 2 - Effect of Diet on Percentage Composition of Oleic Acid

Appendix 3 - Effect of Diet on Percentage Composition of Linoleic Acid
Prostate cancer has been the most diagnosed cancer and the third common cause of cancer death in Canada for years\cite{1}. The medical care for prostate cancer alone in U.S. was $11.85 billion U.S. dollars in 2010 and the number is expected to increase in the next 10 years\cite{2}. Epidemiology studies constantly report high incidence rates of prostate cancer in North America and Northern European countries. In contrast, countries in Asian reported lower incidence rates, which suggested that environmental factors play an important role\cite{3-5}.

Dietary fat was first postulated to be associated with prostate cancer by Armtog and Doll in 1975, and has been studied and confirmed by many researchers since then\cite{6}. As the parent acid of omega-3 fatty acids family, alpha-linolenic acid was suggested by many studies to help to reduce cardiovascular disease risk due to its anti-inflammatory properties\cite{6-8}.

However, the preventive effect was not constantly demonstrated in prostate cancer. A meta-analysis from Brouwer et al. indicated that ALA was related to non-significantly change in coronary heart disease (CHD) risk (RR=0.79, 95%CI: 0.60-1.04), but was associated with 1.7-fold increase of prostate cancer risk (RR=1.70, 95%CI: 1.12-2.58)\cite{9}. Although high heterogeneity was observed in this study, a similar association was also indicated in other meta-analysis, case-control studies and prospective studies\cite{5, 10-15}. This included the Health Professionals Follow-up Study, a large prospective cohort study with 47,866 U.S. health professionals aged 40-75 years old free of cancer at baseline. Food frequency questionnaires were sent every four years for in total of 12 years. Three reports were published from this cohort study, all of which suggested that high ALA intake was significantly associated with increased prostate cancer risk\cite{13, 14, 16}.

There are also many studies indicating that ALA is a protective factor of prostate cancer\cite{17-21}. Meta-analyses focused on prospective studies have shown the negative association between ALA intake and prostate cancer risk\cite{17, 18}. A large case-control study from Europe involving 22,721
cases and 23,034 controls showed weakly reduced prostate cancer risk in participants under 62 years old (OR=0.96, 95%CI: 0.93-0.98)\(^{[21]}\). The metabolism of omega-3 fatty acids as prostate cancer protective nutrients involves their competition with omega-6 fatty acids for enzymes during the process of elongation and desaturation, and inhibition of AA-derived pro-inflammatory eicosanoids and formation of anti-inflammatory eicosanoids.

So far, the intervention studies available in this area are sparse. The Alpha Omega Trial and the Flaxseed Supplementation Study are the only few intervention studies available\(^{[22-26]}\). Results from those studies were again inconsistent: the Alpha Omega Trial and the Flaxseed Supplementation Study showed increased risk while a pilot study from the Flaxseed Supplementation Study showed reduced prostate cancer risk, and one of the pilot studies showed null association. Compared to observational studies, randomized controlled trials are less prone to bias. Attar-Bashi et al. pointed out that more intervention studies were needed in this area, and suggested that future dietary intervention studies could focus on the effect of ALA rich diet on prostate cancer biomarkers\(^{[6]}\).

Prostate specific antigen (PSA) is a serine protease produced by epithelial cells of the prostate gland, and its function is to liquefy seminal fluid\(^{[27, 28]}\). Serum PSA levels are low in healthy subjects, but prostate cancer will stimulate the release of PSA to the blood circulation and result in elevated serum PSA, which is the rationale to use PSA as a prostate cancer biomarker\(^{[29]}\). Although there is controversy on whether PSA should be used as diagnostic standard due to its poor ability to differentiate prostate cancer and other prostatic diseases, PSA is still considered as an important biomarker to predict prostate cancer incidence, tumor growth and recurrence\(^{[30-34]}\).

In addition to the lack of intervention studies, the number of in vitro studies in this area is also small. In clinical trials, the results will be affected by individual variation: age, race, other nutrients intake such as linoleic acid (LA), saturated fat, energy, lycopene and calcium, milk intake, red meat intake, etc. The differences in study populations between studies such as prevalence of PSA screening and lifestyle also make it hard for clinical trials to reach consistent conclusions. In cell culture studies, ALA consistently showed inhibitory effect on prostate cancer cells\(^{[35-38]}\). However, the doses used in most of the cell culture studies were above 100 \(\mu M\), which is hard to reach through the diet\(^{[35-37]}\). Only one study from Motaung et al. used 20 \(\mu M\) and
40 μM of ALA and that study showed suppressed prostate cancer cell growth\textsuperscript{[38]}. It is important to investigate the effect of ALA within physiological range of concentrations on the prostate cancer cell growth, and to connect the findings from in vitro studies with clinical trials.

In the present study, we explore the effect of ALA on prostate cancer through two studies. The first study is a secondary analysis from a 12-week randomized control trial in type 2 diabetes patients to investigate the effect of canola oil enriched bread (rich in ALA) supplementation on serum ALA concentrations and PSA. The second study is an in vitro study to investigate the effect of different ALA concentrations (based on ALA serum concentrations analyzed from the first study) on prostate cancer cell growth and PSA produced from the cells.
2 Literature Review

2.1 Epidemiology of Prostate Cancer

Prostate cancer is the most commonly diagnosed cancer and the third most common cancer worldwide\(^3\). In 2010, the annual net cost for prostate cancer reached $11.85 billion U.S. dollars and the cost is expected to increase in 2020\(^2\). In Canada, prostate cancer accounted for 23.9% of new cancer cases, and was the most frequently diagnosed cancer and the third most common reason of cancer death in males in 2015\(^1\). It is acknowledged that the incidence rate of prostate cancer varies geographically, with higher rates in Northern European and North American countries compared with countries in Asia, such as Japan, India and China\(^3\)\(^-\)\(^5\). Age, family history and ethnicity are confirmed risk factors of prostate cancer. Incidence of prostate cancer increases with age, is higher in patients with family history of prostate cancer, and is higher in Caucasians and African Americans\(^5\),\(^39\),\(^40\). African Americans have the highest incidence rate, which is 50% higher than Caucasians, and 25-fold higher than men from Asia\(^7\),\(^17\). Interestingly, immigrants from low-incidence rate countries experienced an increased incidence rate after moving to high-incidence rate countries\(^7\). Meanwhile, within the same population, increased incidence rate and mortality was found over time. Mortality increased by eight-fold in Japan over a 30-year period and tripled in Hong Kong over a 20-year period, indicating that, apart from ethnicity, environmental factors such as diet might be important factors in development of prostate cancer\(^6\).

2.2 The Role of Dietary fat on Prostate Cancer

In 1975, Armstrong and Doll firstly postulated the relation between dietary fat and death from prostate cancer\(^6\). This hypothesis was tested and confirmed by many researchers, and later on, the research interest turned to specific fatty acids including saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids. However, the results from these studies are inconsistent and conflicting, and vary greatly because of the differences in study design such as study populations, sample sizes, assessment tools and statistical adjustments\(^41\),\(^42\).
2.2.1 Total Fat

There is no consensus on whether fat consumption contributes to an increased prostate cancer risk. A case-control study in Utah revealed a 2.9-fold increased risk among patients who were 68-74 years old and consumed the highest amount of total fat (OR=2.9, 95%CI: 1.0-8.4). But the odds ratio went down to 1.1 and became non-significant when they investigated the association in patients under 68 years old\textsuperscript{[43]}. A positive association was reported again by another study in black patients (OR=2.6, 95%CI: 1.6-4.0)\textsuperscript{[44]}. However, other case-control studies and cohort studies that looked at total fat intake did not find any correlation, and fat intake in a meta-analysis was not linked to prostate cancer risk\textsuperscript{[16, 45-48]}.

2.2.2 Saturated Fat

Ecologic studies showed a positive correlation between saturated fat intake and risk of prostate cancer incidence\textsuperscript{[49-51]}. This association was confirmed in clinical trials. Whittemore et al. studied 1655 prostate cancer patients and 1645 population-based controls in four ethnic groups found that those who consumed the highest amount of saturated fat had two-fold increased prostate cancer risk compared with people in the lowest quintile in all ethnic groups (OR=2.0, 95%CI: 1.2-1.32; P trend<0.01). They also found that the association was significant in Chinese-Americans and Japanese-Americans (P=0.03; P=0.04, respectively) but not in Whites and Blacks\textsuperscript{[52]}. Another case-control study from Hawaii observed that prostate cancer patients had significantly higher saturated fat intake compared with the controls in the group above 70 years old (P=0.02) but not in the group younger than 70 years old. Similarly, saturated fat intake was significantly associated with increased prostate cancer risk in the group above 70 years old (OR=1.7, 95%CI: 1.0-2.8; P trend=0.02), but not in the group younger than 70 years old\textsuperscript{[49]}. Some studies failed to find a significant correlation between dietary saturated fat intake and prostate cancer risk, but no study has reported an inverse correlation\textsuperscript{[40, 46-48, 53]}.

2.2.3 Monounsaturated Fat

No association between monounsaturated fatty acids and prostate cancer risk was observed in cohort studies\textsuperscript{[16, 46]}. A population-based case-control study in Utah with 358 prostate cancer
patients suggested significant increased association between monounsaturated fatty acids intake and total and aggressive prostate cancer risk in males of 68-74 years old but not in males under 68 years old (RR=1.9, 95%CI: 1.1-3.4; RR=3.6, 95%CI: 1.3-9.7, respectively)[43]. However, this study did not adjust for energy intake, and other case-controls studies that adjusted for energy intake failed to detect any significance[40, 47, 48].

2.2.3.1 Omega-6 Polyunsaturated Fat

Omega-6 polyunsaturated fatty acids (PUFAs) have their first double bond at the sixth carbon from the methyl end of the carbon chain exemplified by linoleic acid (LA), gamma linoleic acid (GLA) and arachidonic acid (ARA). LA and ARA are known as precursors of inflammatory eicosanoids and may be related to increased risk of breast cancers[54, 55]. But unlike LA and ARA, GLA was recently reported to have anti-inflammatory properties[56, 57]. Omega-6 PUFAs were not reported to have a significant effect on prostate cancer as a group, however, linoleic acid was reported by some studies to be associated with increased risk[5, 15, 58, 59].

2.2.3.1.1 Linoleic acid

Two studies reported a positive relation between linoleic acid intake and prostate cancer risk [60, 61]. Newcomer et al. measured fatty acids in erythrocyte membranes in both prostate cancer patients and population-based controls, and reported no difference in linoleic acid levels between two groups. But they suggested that subjects in the highest quartile of linoleic acid levels had 2.1 fold higher risk compared with subjects in the lowest quartile (OR=2.1; 95%CI: 0.9-4.8), and the risk of prostate cancer was higher in subjects with higher linoleic acid levels (P trend=0.05)[61]. Godley et al. reported a significant 3.54 fold prostate cancer risk in subjects whose linoleic acid levels were in the highest quartile compared with the lowest quartile, and the risk was positively associated with linoleic acid levels in erythrocyte membranes (OR= 3.54; 95%CI: 1.0-12.53; P trend=0.04)[60].

In contrast, two other studies suggested a protective effect of linoleic acid[20, 39]. A case-control study from Uruguay suggested a negative association between linoleic acid intake and prostate cancer risk and at the same time, a positive association with ALA and prostate cancer risk was
reported. In this study, subjects whose dietary intakes of linoleic acid were at the highest quartile had lower risk compared with whose intake were in the lowest quartile in a multivariate model (OR=0.69; 95%CI: 0.39-1.19; P trend=0.05)\(^{39}\). Another case-control study from Italy suggested a significant decrease of prostate cancer risk in subjects whose consumption of linoleic acid was in the highest quintile (OR=0.8, 95%CI: 0.6-1.0; P trend=0.02).

The majority of the studies failed to find the correlation between linoleic acid and prostate cancer risk\(^{[13, 16, 47, 59, 62, 63]}\). A population-based case-control study in Sweden investigated dietary linoleic acid intake among 526 prostate cancer patients and 536 controls, and showed that the risk of prostate cancer among subjects with higher linoleic acid intake didn't differ from those who consumed less linoleic acid\(^{[47]}\). The same null association was found in a cohort study in Finland, which included 29,133 male smokers after 5-8 years of follow-up\(^{[62]}\). Studies in other countries such as U.S. and Netherlands also reported the same result\(^{[13, 63]}\).

### 2.2.3.1.2 Arachidonic acid

No association of arachidonic acid and prostate cancer risk was observed in cohort studies, including Health Professional Follow-up Study, a cohort study in Sweden, and a cohort study in Netherlands\(^{[13, 59, 63]}\). A biomarker-based case-control study that measured arachidonic acid in erythrocyte membranes or in serum samples also failed to find the correlation\(^{[12, 53, 61, 62]}\).

### 2.2.3.2 Omega-3 Polyunsaturated Fat

Omega-3 polyunsaturated fatty acids (PUFAs) are a group of fatty acids that have the first double bond at the third carbon from the methyl end of the carbon chain exemplified by alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 PUFAs were suggested by many studies as antioxidants with anti-inflammatory properties that had a potential protective effect against many diseases\(^{[64-66]}\). However, studies failed to find significant correlation between omega-3 fatty acids intake and prostate cancer risk\(^{[15, 58, 59, 67]}\). When researchers looked into individual fatty acids, they found that ALA was associated with either increased or decreased prostate cancer risk.
while EPA and DHA were not related to altered risk\textsuperscript{[68]}.

2.2.3.2.1 EPA and DHA

Seafood consumption, which is one of the most important sources of dietary EPA and DHA, was either inversely or not associated with prostate cancer risk in case-control studies. Furthermore, EPA and DHA measured from the tissue samples were not associated with prostate cancer risk. Three case-control studies from Italy and Uruguay showed weak negative association between fish consumption and prostate cancer risk, however none of their results were statistically significant\textsuperscript{[69-71]}. A study in the United Kingdom found that subjects who consumed fish more frequently than meat had significantly lower prostate cancer risk (OR=0.00, 95\%CI: 0.00-0.60)\textsuperscript{[72]}. A study in Poland also reported that either smoked fish or fried fish consumption was associated with decreased prostate cancer risk (OR=0.5, 95\%CI: 0.2-0.8; OR=0.5, 95\%CI: 0.2-0.9, respectively)\textsuperscript{[73]}. Two studies in Japan, where the fish consumption was high, reported an inverse correlation between fish intake and prostate cancer risk (P trend = 0.04, P trend <0.05, respectively)\textsuperscript{[74, 75]}. Studies found lower prostate cancer risk in those who had higher EPA or DHA concentrations in adipose tissue, erythrocyte membranes, or serum compared with those who were in the lowest quartile or quintile \textsuperscript{[53, 60, 61, 76]}.

Several prospective cohort studies suggested fish intake was not related to prostate cancer risk\textsuperscript{[63, 77-80]}. A cohort study in U.S. suggested that subjects consumed fish more than once a week had no significant difference in prostate cancer risk compared to subjects who never regularly had fish (RR=1.57, 95\%CI: 0.88-2.78)\textsuperscript{[77]}. A study in Hawaii focused on people from Japanese ancestry and suggested a similar association in participants consuming fish more than five times a week compared to those who had fish less than one time each week (RR=1.22, 95\%CI: 0.74-2.01)\textsuperscript{[78]}. The only cohort study that reported significantly decreased prostate cancer risk related to increased fish intake was from a Swedish study\textsuperscript{[81]}. After 30 years of follow-up, participants who never or seldom consumed fish had 2.3-fold higher prostate cancer incidence (RR=2.3, 95\%CI: 1.2-4.5; P trend=0.05) and 3.3-fold higher prostate cancer death rate (RR=3.3, 95\%CI: 1.8-6.0; P trend=0.01) compared with those who consumed a high amount of fish.

EPA or DHA were associated with decreased, or not associated with prostate cancer risk in
prospective cohort studies\textsuperscript{[12, 62, 63]}. A cohort study from Netherlands with 58,279 male participants found no increased or decreased prostate cancer risk related to dietary EPA or DHA when comparing the median intake to lowest intake after 6.3 years of follow-up (OR=1.0, 95\%CI: 0.7-1.4; OR=1.0, 95\%CI: 0.8-1.4, respectively)\textsuperscript{[63]}. In studies measuring plasma EPA or total marine fatty acids, no significant correlation was detected as well\textsuperscript{[12, 62]}. However, the Health Professional Follow-up Study reported that high EPA intake was related to decreased total prostate cancer and organ-confined prostate cancer risk (P trend=0.002, P trend=0.03, respectively) but not advanced prostate cancer risk, while DHA was not related to prostate cancer risk\textsuperscript{[13]}. In summary, EPA and DHA were associated with either decreased or not related to prostate cancer risk.

\textit{2.2.3.2.2 Alpha-Linolenic Acid}

ALA is the parent acid of n-3 fatty acids. It can be used to synthesize EPA and DHA through the process of elongation, desaturation and $\beta$-oxidation. LA cannot be synthesized endogenously, which makes it one of the essential fatty acids that can only be obtained through diet\textsuperscript{[82]}. Certain seed oils (canola, flaxseed, chia seed), beans, and walnuts are rich in ALA\textsuperscript{[17, 60]}. In addition to vegetable sources, animal products such as beef, pork and lamb, mayonnaise and butter, and salad dressing are also good sources of ALA\textsuperscript{[16, 67]}. The recommended intake of ALA in Canada is 1.6g/day for males and 1.1g/day for females, however, in many Western countries, the ALA intake is below recommended intake. The deficiency of ALA intake is suspected to be one of the reasons for high prostate cancer incidence\textsuperscript{[3, 83]}.

Many studies suggested that ALA is protective against cardiovascular disease, which is the leading cause of death in males worldwide\textsuperscript{[6-8]}. Brouwer et al. reported that high intake of ALA was associated with decreased fatal heart disease risk (pooled RR=0.79; 95\%CI: 0.60-1.04) after combining risk estimates across 5 prospective cohort studies. However, results from the same research indicated that, despite the health benefit of ALA in heart disease, it was found to increase prostate cancer risk by combining data from 9 observational studies (pooled RR=1.70; 95\%CI: 1.12-2.58)\textsuperscript{[9]}. Despite the high heterogeneity and omission of studies showing null findings, it is surprising to conclude prostate cancer as a possible adverse effect of ALA intake\textsuperscript{[84]}. The debate about whether ALA is protective or harmful to prostate cancer has never stopped.
Results from observational studies are conflicting. Meanwhile, limited evidence from randomized controlled trials and animal studies is available.

2.2.3.2.2.1 Meta-Analysis

ALA was revealed to be associated with increased prostate cancer risk in observational studies. Similar to Brouwer et al., Dennis et al. showed a 26% increase (pooled RR=1.26; 95%CI: 1.10, 1.45) after combining 5 energy-adjusted studies reporting dietary ALA intake in grams of fat\textsuperscript{[10]}. Advanced cancer is proposed to have different etiology compared with localized and low-grade cancer\textsuperscript{[63]}. No association was found between ALA and advanced cancer risk in Dennis et al.'s study\textsuperscript{[10]}. Simon et al. reported a relative risk of 1.20 (95%CI: 1.01-1.43) using data from 8 prospective and 8 retrospective studies\textsuperscript{[11]}. Intriguingly, after they pooled data according to different study designs, significant results were found in retrospective studies (pooled RR=1.51; 95%CI: 0.95-2.39), studies assessing blood or adipose tissue studies (pooled RR=1.54; 95%CI: 1.16-2.06), but not in prospective studies (pooled RR=1.02; 95%CI: 0.88-1.17), or when assessing dietary intake (pooled RR=1.09; 95%CI: 0.91-1.32)\textsuperscript{[11]}.

Studies focusing on prospective studies consistently showed that ALA is not a risk factor for prostate cancer\textsuperscript{[17-19]}. Carayol et al. and Carleton et al. both reported non-significant association between dietary ALA and prostate cancer in prospective studies with significant heterogeneity (pooled RR=0.97; 95%CI: 0.86-1.10; pooled RR=0.95; 95%CI: 0.84-1.09, repectively). The results became significant (pooled RR=0.92; 95%CI: 0.85-0.99; pooled RR=0.91; 95%CI: 0.83-0.99) after removal of Giovannuci et al.'s study, which contributed most to high heterogeneity, and homogeneity was noted as well\textsuperscript{[17,18]}. Carayol et al. pointed out that the food sources of ALA in the early 90s were from unhealthy food like mayonnaise and salad dressings. And it was different from the later known sources of ALA such as flaxseed, canola oil and walnuts. So in Giovannuci's study, which started from the early 90s, food sources of ALA in the early several years might be different from the rest of the period of the study, and also, possibly different from the other studies\textsuperscript{[18]}. Removal of Giovannuci's study was justified and applied by Chua et al. as well, and again, high intake of ALA was related to lower prostate cancer risk (pooled RR=0.915; 95% CI: 0.85-0.99)\textsuperscript{[19]}. By choosing 1.5g/day of ALA intake as cut-point, Carayol et al. further suggested that consuming ALA greater than 1.5g/day was weakly, but
significantly associated with decreased risk of prostate cancer (pooled RR=0.95; 95%CI: 0.91-0.99).

2.2.3.2.2 Prospective Studies

2.2.3.2.2.1 Dietary ALA Intake in Prospective Studies

A non-significant association between ALA intake and prostate cancer risk was found in the Netherlands Cohort Study[63]. They prospectively investigated dietary intake of ALA in 58,279 men. After 6.3 years of follow-up, subjects in highest quintile were less likely to develop prostate cancer compared with those in the lowest quintile (RR=0.76; 95%CI: 0.66-1.04; p trend=0.09). Others studies, like a Multi-ethnic Cohort Study and a prospective study from Sweden detected no protective or stimulating effect associated with ALA intake[58, 59].

Conversely, in the Health Professional Follow-Up Study, a prospective cohort study of 47,866 health professionals with 16 years follow-up, reported increased prostate cancer risk related to high ALA intake in all of their three reports[13, 14, 16]. Of note, the RR reported at the beginning of the study was the highest in the highest quintile compared with the lowest quintile (RR=1.32; 95%CI: 0.82-1.92; p trend=0.04), and after 14 years and 16 years follow-up, the RR dropped by approximately 20% in the same population (RR=1.09; 95%CI: 0.93-1.26; p trend=0.26; RR=1.12; 95%CI: 1.01-1.25; p trend=0.03, respectively)[13, 14, 16]. Moreover, sources of ALA, mostly from mayonnaise and salad dressings, as well as the subjects' life style at the beginning of the study were less healthier than during the later period (such as flaxseed, canola oil and walnuts)[19]. Hence the positive association at the beginning of the study was carried over, and possibly overwhelmed the health benefit of ALA from healthy food.

Discrepant results between studies may arise from confounding factors such as food sources, lifestyle and age. Moreover, the progression of the disease also plays an important role. If the prostate cancer cells are spread outside of prostate gland, it is considered as advanced prostate cancer. In the Health Professional Follow-up Study, ALA was not associated with either total prostate cancer or organ confined prostate cancer, but was related to an almost 2-fold increased risk of advanced prostate cancer (RR=1.98, 95%CI: 1.34-2.93, P trend = 0.001)[13]. Similarly, in the NIH-American Association of Retired Persons (AARP) Diet and Health Study, which
included 288,268 men free of cancer at baseline, no correlation was found in non-advanced cancers, but a weak positive increase in advanced prostate cancer risk was reported (HR=1.17, 95CI: 1.04 - 1.31, P trend=0.01)[42]. It is important for future studies to separate advanced prostate cancer since advanced cancer is suspected to have a different etiology and prognosis[63].

2.2.3.2.2.2.2.2.2.2 Blood ALA Levels in Prospective Studies

Five prospective studies investigated the fatty acid composition of blood samples[12, 53, 62, 85, 86]. Two of the studies did not find the link between ALA levels in blood samples and risk of prostate cancer. This included a nested case-control study in male smokers with 198 prostate cancer cases and 198 controls. They measured cholesterol esters in serum and found that participants with higher proportion of ALA in serum did not have higher prostate cancer risk[62]. A population-based cohort study from Finland with 2,002 middle-aged men and with 12.6 years of follow up measured esterified and non-esterified fatty acids in serum, and suggested that neither was associated with risk of prostate cancer[62, 86].

Two studies showed the positive correlation between high ALA levels in blood and the risk of prostate cancer[53]. Harvei et al. measured serum phospholipids in 141 prostate cancer patients and 282 controls at the baseline when all of the subjects were free of cancer. Their results showed that at the baseline, men that developed prostate cancer had significantly higher phospholipid ALA than cancer-free subjects (P=0.02), and that increased phospholipid ALA was related to increased prostate cancer risk (OR=2.0, 95%CI: 1.1 -3.6)[53]. Gann et al. tested serum cholesterol ester ALA in 120 pairs of case-control subjects from the Physicians' Health Study, a randomized control trial of 14,916 male physicians, and showed that those in the highest quintile had two-fold increased risk of prostate cancer compared with the lowest category (RR=2.14; 95%: 0.93-4.93; p trend=0.03)[12]. Chavarro et al. tested ALA in 476 pairs of subjects from the same study, and showed lower RR of 1.31 (95%CI: 0.89-1.95; p trend=0.24). Of note, the primary objective of the Physicians' Health Study was to look at the effect of aspirin and β-carotene in the prevention of heart disease and cancer[87]. Aspirin in the study was a confounder as a non-steroidal anti-inflammatory drug (NSAID), which is both COX-1 and COX-2 inhibitor and was reported to have an inhibitory effect on prostate cancer[3, 15, 88]. Both reports above included subjects from both the aspirin arm and the control arm, making it possible
that the number of aspirin-treated subjects in each quintile was different from each other, and affected RR in the end.

2.2.3.2.2.3 Case-Control Studies

2.2.3.2.2.3.1 Dietary ALA Intake in Case-Control Studies

One case-control study in Italy, where the main ALA source is olive oil, compared ALA intake between prostate cancer subjects and healthy subjects using food frequency questionnaire. The results showed those in the highest quintile had significantly lower prostate cancer risk compared with those in the lowest quintile (OR=0.7; 95%CI: 0.6-0.9; P trend=0.003), and men with high ALA consumption were less likely to develop prostate cancer (OR=0.84; 95%CI: 0.75-0.94)\cite{20}.

Conversely, another case-control study in Uruguay, which is one of the highest red meat consumption countries (210.4g/day), and where the major source of ALA is from red meat, observed nearly 4-fold increased prostate cancer risk in the highest quintile (OR=3.91; 95%CI: 1.50-10.1; P trend=0.001). Similarly, increased prostate risks related to high ALA consumption were also observed in Swedish and Spanish studies\cite{5,15}.

Of nine case-control studies, five studies did not detect any significant association between ALA consumption and overall prostate cancer, preclinical prostate cancer, and advanced prostate cancer risk\cite{47,67,89-91}. Overall, case-control studies provide weaker evidence, and were more likely to have bias such as recall bias due to the nature of the study compared with prospective studies\cite{11,20,39}.

2.2.3.2.2.3.2 Blood ALA Levels in Case-Control Studies

Most of the case-control studies measured ALA levels in blood showed increased or non-significantly increased risk of prostate cancer in those who had higher ALA levels\cite{60,61,92}. A small study from Korea measured serum phospholipid level in healthy men, benign prostatic hyperplasia patients and prostate cancer patients. Their results showed that mean serum ALA in prostate cancer patients was the highest (2.88±0.33%), followed by benign prostatic hyperplasia patients (2.21±0.21%), and healthy males (1.97±0.28%), and the trend was significant
Newcomer et al. tested ALA levels in erythrocyte membranes in prostate cancer patients and population-based controls. The results showed a 2.6-fold increased risk in the highest versus the lowest quintile (OR=2.6; 95%CI: 1.1-5.8; P trend=0.01)\textsuperscript{[61]}. Godley et al. tested ALA in the same tissue, but the results did not reach statistical significance (OR=1.69; 95%: 0.54-5.26), and the overall trend was not significant\textsuperscript{[60]}. Adipose tissue is a better biomarker for long-term ALA intake compared with erythrocyte membranes\textsuperscript{[3,10,93]}. Godley et al. also measured fatty acid composition in adipose tissue, and showed a non-significant association in prostate cancer risk associated with increased to ALA concentration in tissue (OR=2.73; 95%CI: 0.70-10.61; P trend=0.18)\textsuperscript{[60]}.

The only study that suggested a possible protective effect of ALA was from Freeman et al\textsuperscript{[94]}. Subjects with localized prostate cancer and awaiting radical prostatectomy were enrolled. Of note, they measured prostatic fatty acid composition in target tissue and found that prostatic ALA was lower in stage T3 tumor, or in the presence of perineural invasion, compared with less aggressive category (P= 0.02 to 0.049)\textsuperscript{[94]}.

2.2.3.2.2.4 Intervention Studies

Data from randomized controlled trials are very sparse, with only four reports derived from two studies available\textsuperscript{[22-24,95]}. The Alpha Omega Trial was a double-blind, placebo-controlled trial that primarily looked at the effect of omega-3 fatty acids on cardiovascular disease. Subjects were provided with ALA enriched margarine (2g ALA), or EPA and DHA enriched margarine (400mg EPA and DHA), or both, or a placebo margarine everyday for 40 months. Daily intake of 2g ALA through margarine resulted in an increase of 0.11ng/ml in PSA compared to the controls at the end of the study. However, the 95% confidence interval included no effect (mean=0.11; 95%CI: -0.09-0.32ng/ml)\textsuperscript{[22]}. The Flaxseed Supplementation study was a randomized controlled trial looking at the effect of flaxseed supplementation and/or dietary fat restriction on prostatic carcinoma\textsuperscript{[26]}. Patients with biopsy-confirmed prostate cancer awaiting prostatectomy received flaxseed supplementation (flaxseed 30g/day), or a low-fat diet (≤20% of total energy from fat intake), or both, or on a usual diet\textsuperscript{[26]}. Each gram of flaxseed in the study contained 0.217g ALA, thus the flaxseed arm
received 6.5g ALA every day\textsuperscript{105}. In a pilot study, 15 patients awaiting repeat biopsy were on a fat-restricted, flaxseed-supplemented diet. Contrary to results from Alpha Omega Trial, decreased total serum PSA and decreased benign epithelial cell proliferation rates were observed after 6 months compared with their baseline (P=0.0002; P=0.0168, respectively)\textsuperscript{24}. However, another pilot study on 25 prostate cancer patients failed to find significant change in PSA (P=0.10)\textsuperscript{23}. The flaxseed supplementation study on 161 men showed no difference of ALA levels in prostatic tissue between treatment and placebo group, but prostatic ALA was significantly positively associated with PSA (P=0.028). However, flaxseed is also a major source of lignan, which showed antioxidant and anti-angiogenic effects on prostate cancer. Thus, it is hard to separate the effect of ALA from lignan in the study\textsuperscript{25}.

### 2.2.3.2.2.5 Cell Culture Studies

ALA showed inhibitory effects on the growth of prostate cancer cell lines in many studies\textsuperscript{35-38}. In Eser et al.'s study, both androgen-dependent PC-3 cells and androgen-independent LNCaP cells treated with 100\(\mu\)M of ALA for 4 days showed decreased cell growth over the time\textsuperscript{35}. The decreased cell growth was not due to increased apoptosis, but was associated with down-regulated fatty acid synthase (FASN), a synthase related to prostate carcinogenesis, through decreased sterol response element binding protein-1 (SREBP-1), cell cycle arrest, and decreased inflammation\textsuperscript{35}. Prinsloo et al. found that DU-145 cells treated with 100\(\mu\)M ALA noncompetitively inhibited testosterone binding to prostate cancer cells, and this effect was related to reduced androgen receptor binding capacity. Also, the inhibitory effect was more pronounced when the dose increased to 200\(\mu\)M\textsuperscript{36}. Liu et al. showed the inhibitory effect of ALA on prostate cancer cell proliferation started from 100\(\mu\)M in medium containing testosterone or 5\(\alpha\)-dihydrotestosterone, and from 500\(\mu\)M in regular cell culture medium. The effect was most likely from inhibition of 5\(\alpha\)-reductase. However, the possibility of direct cytotoxicity could not be ruled out\textsuperscript{37}. Motaung et al. found that ALA significantly stimulated cell death at 20\(\mu\)M and 40\(\mu\)M, and the percentage of dead cell continued to increase along with the concentration (\(R^2=0.6361, P=0.0019\))\textsuperscript{38}.

### 2.2.3.2.2.6 Animal Studies
ALA does not exert significant effects on prostate tumor growth in animal studies [96, 97]. Connolly et al. established a prostate cancer animal model by injecting DU-145 human prostate cancer cell lines under the skin of athymic nude mice. They then observed an increased percentage of ALA in the tumor phospholipid fraction in linseed oil fed mice compared with mice on a corn oil or menhaden oil diet, while no significant difference was observed in tumor surface area between groups, indicating that the ALA enriched diet neither stimulated nor suppressed the growth of DU-145 cell tumors in nude mice [96]. Similar results were found by Mori et al. when they induced prostate cancer in F344 rats by 3,2'-dimethyl-4-aminobiphenyl (DMAB) and testosterone propionate (TP). Feeding a parilla oil enriched diet did not influence the incidence of prostate intraepithelial neoplasia or prostate carcinoma in groups given DMAB only. However, a decreased incidence was observed in groups given DMAB and TP and fed a parilla oil diet [97].

2.3 Possible Mechanisms

2.3.1 Eicosanoid synthesis

Eicosanoids, including prostaglandins, hydroxyeicosatetraenoic acids (HETEs) and leukotrienes, are potent biochemical compounds generated from polyunsaturated fatty acids, and play an important role in regulating inflammation, which is closely related to the incidence and progression of many cancers such as lung, breast, colorectal, and prostate cancer [98-102]. Pro-inflammatory eicosanoids are arachidonic acid derived compounds that can stimulate inflammation and carcinogenesis, while anti-inflammatory eicosanoids are eicosapentaenoic acid derived compounds that help to reduce inflammation [15, 67, 103].

The synthesis of pro-inflammatory eicosanoids begins with the release of AA from membrane phospholipids through phospholipase A$_2$ [104]. Both AA and EPA can be synthesized endogenously through the process of desaturation and elongation by ∆-6 desaturase, ∆-5 desaturase and elongase enzymes from their precursors LA and ALA (Figure 1) [82, 92]. Increased ALA intake competes with LA for rate-limiting enzymes, and thus restricts the formation of AA and cuts down the amount of substrates available for pro-inflammatory enzymes. Meanwhile, increased levels of EPA produced by ALA metabolism compete AA for phospholipase A2 in membrane phospholipids, limiting AA from being mobilized.
The synthesis of prostaglandins starts from conversion of precursor fatty acids to prostanoids (PGs) by the catalytic action of cyclooxygenase (COX) and peroxidase[105] (Figure 2). Through the COX pathway, AA is converted to prostaglandin E₂ (PGE₂), thromboxane A₂ (TXA₂), and prostacyclin I₂ (PGI₂). PGE₂ promotes tumor growth by stimulating tumor cell proliferation, inhibiting apoptosis, promoting tumor angiogenesis and inducing tumor invasion. It also induces cyclooxygenase-2 (COX-2) synthesis, a COX isoform that can be barely found in normal cells but is overexpressed in prostate cancer cells[101, 103, 106]. Inhibitors of COX, such as non-steroidal anti-inflammatory drugs (NSAIDs) were shown by many studies to be associated with decreased prostate cancer incidence and mortality[107]. EPA generated from ALA, as well as from dietary intake, inhibits COX-2 by competing with AA for the COX pathway. Instead of generating the COX-2 stimulator PGE₂, EPA is converted to prostaglandin E₃ (PGE₃), thromboxane A₃ (TXA₃), and prostacyclin I₃ (PGI₃)[3, 4, 106]. Moreover, PGE₃ inhibits tumor cell growth by inhibiting proliferation, angiogenesis and invasion, possibly through activation of PG receptors E types (EP) receptors[108]. Therefore, ALA could inhibit prostate cancer possibly by producing EPA, inhibiting the activity of COX-2 and suppressing the synthesis of pro-inflammatory eicosanoids.

The other pathway that is involved in eicosanoid synthesis is through lipoxygenase (LOX) (Figure 3). AA is converted to 5-HETE (5-hydroxyeicosatetraenoic acid), 12-HETE and 15-HETE by the activity of 5-LOX, 12-LOX and 15-LOX respectively[103]. 12-HETE has been reported to promote cancer by promoting angiogenesis, increasing cancer cell survival and metastasis, and was associated with regulating prostate cancer progression[109, 110].

5-LOX converts AA to 5-HPETE (hydperoxyeicosatetraenoic acid), which reduces to 5-HETE, or is converted by lipoteichoic acid (LTA) synthase to leukotriene (LT) B₄, LTC₄, LTD₄, and LTE₄. Studies showed that inhibition of 5-LOX induced apoptosis and suppressed expression of 5-HETE, and this effect was associated with reduced prostate cancer cell growth[111-114]. EPA competes with AA with 5-LOX and 12-LOX, reduces production of pro-inflammatory eicosanoids, and generates 5-HEPE, 12-HEPE and LTB₅, LTC₅, LTD₅ and LTE₅, which are not associated with prostate cancer promotion. Although the competition of 15-LOX with AA results in decreased anti-inflammatory 15-HETE levels, the metabolite produced by EPA was reported to suppress prostate cancer growth as well[115]. So the competition for 12-LOX and 5-LOX by
ALA during its metabolism will inhibit the synthesis of cancer related metabolites and suppress prostate cancer.

Intriguingly, unlike other HETEs, 15-HETE, which is produced by 15-LOX2, enhances apoptosis and has anti-inflammatory properties that were associated with suppressed prostate tumor growth[116-118]. An increase in ALA suppresses the production of AA and thus reduce the synthesis of anti-carcinogenic 15-HETE might be one of the reasons that ALA might increase prostate cancer risk.

### 2.3.2 Free radical formation

One of the postulated mechanisms by which ALA promotes prostate cancer is through the increased formation of free radicals[5, 7, 18]. The double bonds in ALA are vulnerable to oxidation, and form lipid hydroperoxides, which will generate more oxygen radicals (Figure 4). Additional oxygen radicals will either damage DNA, or attack more ALA and form a vicious cycle[68]. Oxidized ALA in cell membranes is removed by phospholipase A2 to sustain the membrane's function, however, the activation of phospholipase A2 will release AA from membrane, initiate pro-inflammatory eicosanoids, and stimulate carcinogenesis. Phospholipase A2 and its byproduct lysophosphatidylcholine were reported by many studies to be involved in prostate cancer development and progression[119-122].

### 2.4 Prostate specific antigen as a biomarker of prostate cancer

Prostate specific antigen, also known as gamma-seminoprotein or kallikrein-3 (KLK3), is a kallikrein-like serine protease encoded by KLK3 gene and produced by epithelial cells of the prostate gland[27]. The biological function of PSA is to liquify seminal fluid[28]. In healthy subjects, the rate of release of PSA from seminal plasma into the serum is very low. PSA detected from healthy subjects' plasma should be lower than 4 ng/ml, which is much lower than the concentration of PSA in seminal plasma (0.5-5 mg/ml)[29]. In prostate cancer patients, the release of PSA to serum is enhanced and the PSA detected is much higher, which is the rationale for PSA screening. Compared to digital rectal examination, PSA screening has improved sensitivity and is no longer affected by variation between examiners, which has helped it to gain
popularity. However, elevated PSA in serum is specific to prostate cancer. Other prostatic diseases such as prostatitis and benign prostatic hyperplasia can also result in increased PSA in serum\textsuperscript{[29]}. The major concern is that PSA screening fails to differentiate prostate cancer and other prostate diseases and results in an elevated number of unnecessary biopsies and increased health care costs: subjects with serum PSA level above 4 ng/ml are always recommended to have a prostate biopsy, 60-80\% of which result in no prostate cancer\textsuperscript{[29, 123]}.

Also, there is no consensus on whether PSA screening can reduce prostate cancer mortality. In order to evaluate the effectiveness of PSA screening on prostate cancer, many clinical trials including two large clinical trials: PLCO (Prostate, Lungs, Colorectal and Ovarian) and ERSPC (the European Randomized Study of Screening for Prostate Cancer) have been established\textsuperscript{[124-127]}. In the PLCO study, 76685 men from the U.S. were enrolled and randomized to either the intervention arm with annual PSA testing and DRE, or control arm with usual care. After 13 years follow-up, the intervention arm had a significant 12\% relative increase in prostate cancer incidence (RR=1.12, 95\%CI: 1.07-1.17), but no significant difference of high-grade prostate cancer incidence and mortality from prostate cancer was detected\textsuperscript{[125]}. In the ERSPC study, 182,000 men in seven European countries were randomized to a screening group, which received PSA screening every 4 years, or a control group. After 13 years follow-up, they also found an increased prostate cancer incidence in screening group compared to the control arm (RR=1.57, 95\%CI: 1.51-1.62), but at the same time, significant decreased prostate cancer mortality was reported (RR=0.79, 95\%CI: 0.69-0.91, P=0.001). A systematic review pooled six randomized controlled trials and showed that PSA screening significantly increased prostate cancer diagnosis (RR=1.46, 95\%CI: 1.21-1.77) and non-significantly decreased prostate cancer death (RR=0.88, 95\%CI: 0.71-1.09)\textsuperscript{[128]}.

Despite the controversy in PSA screening as a diagnosis standard and its lack of ability to improve prostate cancer mortality, PSA screening is still useful in predicting prostate cancer incidence and recurrence\textsuperscript{[30-34]}. A prospective study from Gann et al. reported that baseline PSA correctly predicted 85\% (95\%CI: 74-95\%) of aggressive prostate cancer occurring during the following four years\textsuperscript{[30]}. Ulmert et al. also found that PSA levels were significant increased before clinically advanced prostate cancer was diagnosed and concluded that a PSA test before the age of 50 was a strong predictor of advanced prostate cancer\textsuperscript{[32]}. MacKitosh et al. studied
prostate cancer death and PSA levels at the diagnosis on 230,081 patients and suggested that high PSA levels prior to diagnosis indicated high prostate cancer mortality (p<0.0001)\textsuperscript{[31]}. Inoue et al. suggested that PSA levels 3 months after radical prostatectomy (3M-PSA) independently predicted the survival of patients without biochemical recurrence (hazard ratio: 7.977, 95%CI: 3.552-17.913, P<0.001)\textsuperscript{[33]}. These studies suggested that although PSA screening may be in debate, the role of PSA as a predictor and a surveillance tool of prostate cancer still makes it an important clinical measure.

### 2.5 Effect of Type 2 Diabetes on Prostate Cancer

Many studies have found that type 2 diabetes patients have a lower risk of prostate cancer\textsuperscript{[129-133]}. A national cohort study from Sweden investigated the risk of prostate cancer in 198,129 male physicians with a family history of type 2 diabetes, and compared to matched controls. They found a decreased risk of prostate cancer in men with a family history of type 2 diabetes (standardized incidence ratio: 0.87, 95%CI: 0.86-0.89)\textsuperscript{[129]}. Similarly, another population-based cohort study in Israel reported reduced hazard ratios for prostate cancer in people with type 2 diabetes (HR=0.5, 95%CI: 0.56-0.74)\textsuperscript{[130]}. A meta-analysis of observational study pooled the results from 29 cohort studies and 16 case-control studies, and found a significant inverse relationship between type 2 diabetes and prostate cancer risk (RR=0.86, 95%CI: 0.80-0.92)\textsuperscript{[134]}. It has been suggested by many studies that the anti-diabetic drug, metformin, was associated with reduced prostate cancer risk and the reduced prostate cancer biomarker, PSA\textsuperscript{[135-137]}. Jayalath et al. conducted a cross-sectional study in 326 type 2 diabetes patients without prostate cancer. They found that PSA was reduced by 8\% (95%CI: 2\% to 13\%, P=0.011) per 500-mg/d increase in metformin\textsuperscript{[135]}. A study from Chong et al. pointed out that metformin significantly reduced prostate cancer death, recurrence, metastases, secondary cancers in prostate cancer patients with type 2 diabetes (P<0.004)\textsuperscript{[136]}. In vitro studies have shown similar results. Metformin had inhibitory effect on prostate cancer cell growth\textsuperscript{[138-140]}. However, some studies did not find the significant association between metformin use and prostate cancer risk\textsuperscript{[141,142]}. This included a meta-analysis of six cohort studies and four case-control studies. They reported that the overall association was not significant (RR=0.92, 95%: 0.84-1.02, P=0.112). However, when they found
patients treated with metformin showed a significantly reduced risk of prostate cancer in cohort studies (RR=0.92, 95%CI: 0.87-0.96, P<0.010), but not in case-control studies (RR=0.95, 95%CI: 0.78-1.16, P=0.632).

The other possible reason that type 2 diabetes patients have a lower prostate cancer risk is related to hypoinsulinemia in some patients due to the dysfunction of pancreatic β-cell. Insulin is reported to stimulate prostate cancer growth\textsuperscript{[143]}. Hypoinsulinemia in some type 2 diabetes patients may be the result of increased plasma level of insulin-like binding protein 1 (IGFBP-1), and thus decreased insulin-like growth factor 1 (IGF-1) synthesis\textsuperscript{[144, 145]}. Increased IGF-1 has been shown to stimulate prostate cancer cell growth and increase prostate cancer risk, and inhibition of IGF-1 has been shown to reduce prostate cancer tumor growth\textsuperscript{[146-149]}. Also, insulin was reported to be positively associated with bioavailability of testosterone levels, which is a stimulating factor for prostate cancer\textsuperscript{[150, 151]}. Therefore, hypoinsulinemia in some type 2 diabetes patients have lower testosterone.
**Figure 1** - Metabolism of n-6 and n-3 fatty acids

**Figure 2** - Synthesis of eicosanoids through COX pathway
Figure 3 - Metabolism of AA and EPA through LOX pathway

Figure 4 - The effect of oxidation on ALA
3 Hypothesis and Objectives

3.1 Hypothesis

General:
Our hypothesis is that ALA will inhibit prostate specific antigen (PSA) levels.

Specific:

1. An ALA-enriched diet will lower serum PSA levels in and increase serum ALA levels in type 2 diabetes patients.

2. Increased concentrations of ALA will be associated with decrease cell growth and PSA production in vitro.

3. This in vitro effect will be specific for ALA.

3.2 Objectives

General:

The objective of the study is to assess the effect of ALA on prostate cancer biomarker.

Specific:

1. To determine the effect of ALA-enriched diet on serum PSA levels and serum ALA levels in type 2 diabetes patients.

2. To assess the effect of ALA at different concentrations on prostate cancer cell growth and PSA in vitro.

3. To compare the effect of ALA with other fatty acids in vitro.
Chapter 4
Secondary Analysis from a Randomized Control Trial: The Effect of Alpha-Linolenic Acid Supplementation on Serum Prostate Specific Antigen

4 Secondary Analysis from a Randomized Control Trial: The Effect of Alpha-Linolenic Acid Supplementation on Serum Prostate Specific Antigen

4.1 Abstract

Background: Prostate cancer is the second most common cause of cancer death. Consumption of ALA has increased in the past decades. As the parent fatty acid of n-3 fatty acids, it is related to anti-inflammatory properties, and was reported to benefit coronary heart disease. However, it has been implicated in prostate cancer, where most of the results are come from observational studies. Few intervention studies are available.

Objectives: To assess the effect of ALA on prostate specific antigen (PSA) from a randomized control trial. To assess the effect of ALA on serum lipid fractions. To explore the relationship between change in each ALA fraction and change in PSA.

Methods: 141 subjects with type 2 diabetes were randomized to receive either canola oil-enriched whole wheat bread or high wheat fiber bread as a supplement everyday for 12 weeks. PSA was measured in male participants (35 control and 29 test). Fasting serum at screening, week 0, week 4, week 10 and week 12 was collected and used for fatty acids analysis, in total lipids, free fatty acids, phospholipids and triglycerides fractions.

Results: Consumption of ALA-enriched bread for 12 weeks PSA was reduced by 0.43 ng/ml in the test group compared to controls (P=0.014). Increased percentage composition of total lipid ALA, free fatty acid (FFA) ALA, phospholipid ALA and triglyceride ALA in serum was observed in test group (P<0.05). The concentration of total lipid ALA and phospholipid ALA in serum was increased in test group compared to the controls (P=0.015, P=0.035, respectively). Change in PSA was not associated with change in ALA after removal of outliers.

Conclusion: This study suggested that supplementation of ALA decreased PSA and increased the percentage composition of ALA in all lipid fractions in type 2 diabetes patients.
4.2 Introduction

Prostate cancer is the second most common cause of cancer death in men\textsuperscript{[7]}. The incidence rates of prostate cancer vary geographically, indicating environmental factors such as diet may have an impact on the development of the disease\textsuperscript{[18, 68]}. ALA, as the parent fatty acid of the n-3 fatty acids, has been shown by many studies to have a protective effect in coronary heart disease due to its anti-inflammatory properties\textsuperscript{[6-8]}. The consumption of ALA has increased >100-fold, and the availability of ALA has been increased by 40\% during recent decades\textsuperscript{[118, 152]}. However, this increase does not appear to have had an effect on prostate cancer, with a number of studies showing either stimulating effects, or protective effects, or no effect\textsuperscript{[10, 11, 18, 19, 63]}. The majority of these results come from observational studies, which are more susceptible to biases such as selection bias, information bias, recall bias, and confounding\textsuperscript{[153]}.

Only 4 papers from randomized controlled trials are available, the results of which were also inconsistent\textsuperscript{[22-24, 95]}. The Alpha Omega Trial showed a non-significant change in PSA after taking extra 2g of ALA every day for 40 months\textsuperscript{[22]}. Azrad et al. showed a significant increase in PSA when comparing subjects supplemented with flaxseed to controls\textsuperscript{[95]}. Two pilot studies of flaxseed supplementation showed either decreased risk or a null association\textsuperscript{[23, 24]}.

The main purpose of this study is to explore the effect of ALA on prostate cancer biomarker, PSA, by using data and serum samples from a randomized parallel study on type 2 diabetes patients. The additional purpose of the study is to explore the effect of feeding ALA-enriched diet on serum ALA concentrations, and the correlation between change in PSA and change in ALA fractions.

4.3 Methods

4.3.1 Study Participants and Protocol for Original Study
423 subjects were recruited from newspaper, public transport and hospital clinical advertisements for screening. Inclusion criteria were patients with a type 2 diabetes history for more than 6 months, were on a stable dose oral antihyperglycemic medication for at least the previous two months, and HbA1c values were between 6.5% and 8.5%. Exclusion criteria included participants with clinically significant cardiovascular, renal (creatinine higher than 150μmol/L) or liver disease (ALT more than 3 times the upper limit of normal) or with a history of cancer\textsuperscript{[154]}. After screening, 282 participants were excluded for multiple reasons given in Figure 1 and 141 participants were included in randomization. 70 participants were randomized to the test group and 71 participants were randomized to the control group.

Participants visited the Risk Factor Modification Centre of St. Michael's Hospital for screening at week 0, 2, 4, 8, 10 and 12 (end of the study). Fasting Blood samples were taken, and weight, waist circumference, and seated blood pressure were measured during the visit. Self-administered seven-day food records that described diet for 7 days prior to the visit were provided by participants, and were reviewed by a dietitian. Dietary advice was given at the same time. Supplementary bread was picked up the same day.

4.3.2 Dietary Intervention

The test group received dietary advice to consume a low glycemic index diet, and was provided with 4.5 slices of canola oil-enriched whole wheat bread (500kcal/day) providing 2.67g ALA as a supplement every day. The control group was advised to consume a high wheat fiber diet, and was provided with 7.5 slices of whole wheat bread without canola oil (500kcal/day) providing 0.11g ALA every day (Table 1).

4.3.3 Study Population and Protocol for the Current Study

Male participants from 141 eligible subjects were included in the study. Exclusion criteria included participants dropped out the trial, did not provide fasting blood samples at baseline (screening or week 0), or week 12, didn't have PSA data at baseline, or week 12 and had PSA greater than 4ng/ml at baseline. Participants who provided blood samples with both baseline and week 12, but missed week 4 or week 10 blood samples were still included in the study. In total
35 men from control group and 29 men from test group were available for the analysis. At screening, week 0, week 4, week 10 and week 12, fasting blood samples were used for PSA measurement and fatty acids analysis (Figure 5).

4.3.4 PSA measurement

Fasting blood samples were taken by a nurse, and serum was centrifuged and collected by a trained technician. Serum samples were divided into several aliquots and one of them was sent to clinical laboratory on dry ice for PSA measurement. PSA from the serum was analyzed using the Hybritech PSA Assessing Assay, a two-site immunoenzymatic assay.

4.3.5 Fatty Acids Analysis

4.3.5.1 Internal Standard Preparation

The internal standard was made by dissolving heptadecanoic acid (17:0; Nu-chek Prep, Waterville, Minnesota, U.S.), triheptadecanoin (17:0 TG), cholesteryl heptadecanoate (17:0 CE) and 1, 2-diheptadecanoyl-sn-glycero-3-phosphocholine (17:0 PC; Avanti Polar Lipids, Alabaster, Alabama, U.S.) in chloroform. The concentrations of lipid fractions were 10 ug/mL for FFA C17:0, 20 ug/mL for CE C17:0, 75ug/mL for TG C17:0, 100 ug/mL for PC C17:0.

4.3.5.2 Extraction of lipids

Serum samples from the above were stored in -80°C freezer. At the time of analysis, serum samples were thawed on the bench to room temperature. Lipids from the serum were extracted by Folch method. Three hundred micro mole of serum along with 300 μl heptadecanoic acid (C17:0) internal standard were homogenized in 7.6ml chloroform, methanol and potassium chloride at the ratio of 4:2:1.6. The mixture was separated by centrifuged at 1460 revolutions per minute (RPM) for 10 minutes. The bottom clear chloroform phase containing lipids was extracted. With the leftover mixture, lipid was extracted again through the same steps. Samples
were dried in a nitrogen evaporator and reconstituted in chloroform. This was divided to two aliquots. One was used for measuring total lipids, and the other was used for measuring lipid fractions measurement.

4.3.5.3 Separation of Lipid Fractions

Lipid fractions were separated by thin layer chromatography (TLC). TLC plates were scored and pre-washed in chloroform/methanol (2:1) overnight, and were activated at 100°C oven for an hour. The reconstituted fraction was dried down in nitrogen evaporator and was loaded onto the TLC plates (6 samples per plate). Three 1 cm bands were loaded with the internal standard for reference. Plates were placed in a clear tank containing heptane/diethyl ether/glacial acetic acid (60:4:2) mixture until migration was up to 2 cm from top. Plates were dried in a fume hood and sprayed with 0.1% of ANSA (8-Anilino-1-naphthalenesulfonic acid ammonium salt). Phospholipids (PL), free fatty acids (FFA), triglycerides (TG) and cholesterol esters (CE) were visualized and scored under the UV light. Each lipid fraction was collected and transferred to 15ml glass tubes. 2ml hexane was added to each tube and tubes were filled with nitrogen and stored in an -80°C freezer.

4.3.5.4 Preparation of Fatty Acid Methyl Esters (FAMEs)

Total lipids, PL, FFA, TG and CE were methylated at 100°C with 14% boron trifluoride in methanol, and FAMEs were extracted with hexane for an hour. The methylation was stopped by adding dH₂O and the mixture was separated by centrifuge (1460 rpm for 10 minutes). The top layer containing hexane and fatty acids was transferred to a new GC vial and was dried under nitrogen. The fatty acids were reconstituted with hexane.

4.3.5.5 Gas Chromatography

FAMEs were analyzed by gas chromatography-flame ionization detection (GC-FID), using Varian-430 gas chromatograph (Varian, Lake Forest, California, U.S.) with a Supelco capillary column (SP-2560; 100 m × 0.25 mm i.d. × 0.20 μm film thickness; Sigma-Aldrich, Toronto, Ontario). The injector and detector were set at 250°C. Samples were analyzed in splitless mode.
4.3.5.6 Calculation

4.3.5.6.1 The Concentrations of Fatty Acids

The concentrations of fatty acids were calculated by comparison with the peak area of the C17:0 standard added at the beginning of the extraction, and was expressed as $\mu$mol/L ($\mu$M).

4.3.5.6.2 The Percentage Composition of Fatty Acids

The percentage composition of fatty acid normalized to 100% was calculated by dividing peak area of an individual fatty acid by the total peak area of 22 fatty acids.

The 22 fatty acids were as follows: lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1n-7), palmitic acid (C16:0), palmitoleic acid (C16:1 n-7), stearic acid (C18:0), oleic acid (C18:1 n-9), cis-vaccenic acid (C18:1 n-7), linoleic acid (C18:2 n-6), gamma-linolenic acid (C18:3 n-6), alpha-linolenic acid (C18:3 n-3), arachidic acid (C20:0), eicosanoic acid (C20:1 n-9), eicosadienoic acid (C20:2 n-6), eicosatrienoic acid (C20:3 n-3), arachidonic acid (C20:4 n-6), behenic acid (C22:0), eicosapentaenoic acid (C20:5 n-3), adrenic acid (C22:4 n-6), docosapentaenoic acid (C22:5 n-6), docosapentaenoic acid (C22:5 n-3), docosahexaenoic acid (C22:6 n-3), nervonic acid (C24:1 n-9).

4.3.6 Statistical Analysis

Results were expressed as mean ± standard error (SE). Baseline was calculated by taking the average of values from screening samples and week 0. If data from the screening sample was missing, week 0 was used as baseline, and vice versa. Student's 2-tailed t-test was used to compare the difference between treatments at specific time points. The differences in treatments over time were taken from a repeated measures model in SAS 9.4, modeling end values from week 4, 10 and 12, and using baseline as a covariate. The model had an unstructured covariance structure grouped by randomization in order to adjust for the unequal variance seen between groups. Pearson correlations were used to determine the correlation between change in PSA and change in ALA. P values < 0.05 were accepted as significant.
4.4 Results

4.4.1 Baseline Characteristics

Baseline characteristics of the participants were presented in Table 2. No significant difference was found at baseline.

4.4.2 Consumption of Bread

The control group was offered 214g of high wheat fiber bread every day (500 kcal), and consumed 85.97%. And the test group was offered 133g of canola oil enriched bread every day (500 kcal), and consumed 98.84%.

4.4.3 Sample Collection

All study subjects' samples were available at baseline, with 35 samples for control group and 29 samples for test group. Five subjects (control group) from week 4, 6 samples (5 from control and 1 from test) from week 10 and 6 subjects (2 from control and 4 from test) were missing due to the absence of the visit or missing PSA data. In sum, 92.578% of blood samples were available for the analysis.

4.4.4 Prostate Specific Antigen (PSA)

At baseline, the mean PSA level was 1.5±0.2 ng/ml for controls (n=35), and 1.2±0.2 ng/ml for test subjects (n=29). No significant difference was detected between test and control at baseline. No significant difference in PSA was observed for controls over the time (n=35). During the intervention, PSA was decreased by 0.2 ng/ml for the test subjects compared to its baseline (P=0.001). PSA level in the treatment group was reduced by 0.43 ng/ml during the study compared to the controls (P=0.014) (Figure 5).

4.4.5 Serum Total lipid ALA
4.4.5.1 Percentage Composition of Total Lipid ALA

Percentage composition of serum total lipid ALA at baseline was not different between groups: 0.84±0.05% for controls (n=35), and 0.86±0.27% for the test group (n=29). During the study, total lipid ALA was significantly decreased by 0.08% in the control group (P=0.012), and was significantly increased by 0.25% in the test group (P=0.001). The test group had significantly 0.33% higher serum total lipid ALA compared with the control group during the intervention (P<0.001) (Figure 6a).

4.4.5.2 Concentrations of Total Lipid ALA

Average total lipid ALA concentration in serum was 32.26± 2.49 μM (μmol/L) for controls and 36.39±5.13 μM for test subjects at baseline. No significant difference was found between groups at baseline. During the study, total lipid ALA in the control group was decreased by 3.91 μM (P=0.029), while no significant change was found in the test group. ALA level was increased by 9.60 μM in the test group compared to the control group during the study (P=0.015) (Figure 7a).

4.4.6 Serum Phospholipid ALA

Among all the serum samples available (n=237), data from 1 subject in the control group at baseline, 3 subjects in the test group from week 4, 1 subject in the control group from week 10 and 3 subjects in the control group from 12 were missing due to the contamination of samples. In total, 96.624% samples were analyzed.

4.4.6.1 Percentage Composition of Phospholipid ALA

No significant difference was observed in serum phospholipid ALA at baseline between groups, with 0.20±0.01% for controls, and 0.19±0.02% for test subjects. Serum phospholipid ALA did not change significantly over the time in the control group. The test group had an increase of 0.05% in phospholipid ALA during the intervention compared to its baseline (P=0.001). ALA supplementation resulted in an increase of 0.06% in serum phospholipid ALA in the test group compared to controls (P=0.001) (Figure 6b).
4.4.6.2 Concentrations of Phospholipid ALA

At baseline, serum phospholipid ALA was 2.89±0.12 \( \mu \text{M} \) for controls and 2.81±0.37 \( \mu \text{M} \) for test subjects. Serum phospholipid ALA was not different between test subjects and controls at baseline. No significant within group change was observed during the study in either test or control group. During the study, the test group had an increase of 0.61 \( \mu \text{M} \) phospholipid ALA levels when compared to the control group (\( P = 0.035 \)) (Figure 7b).

4.4.7 Serum Triglyceride ALA

Among all the serum samples available (n=237), data from 3 subjects (1 from control and 2 from test) from week 4, 2 subjects (1 from control and 1 from test) from week 10 and 2 subjects in the control group from 12 were missing due to the contamination of samples. In total, 97.046% samples were analyzed.

4.4.7.1 Percentage Composition of Triglyceride ALA

Triglyceride ALA in the test group (0.94±0.10%) was not different from controls (0.99±0.09%) at baseline. Serum triglyceride ALA did not change significantly in controls during the study, but was increased by 0.28% in the test group (\( P=0.011 \)). The test group had an increase of 0.27% of triglyceride ALA over the time compared with the control group (\( P= 0.026 \)) (Figure 6c).

4.4.7.2 Concentrations of Triglyceride ALA

Average triglyceride ALA concentration in serum was 7.25±1.01 \( \mu \text{M} \) for controls and 8.38±1.54 \( \mu \text{M} \) for test subjects at baseline. No significant difference was found between groups at baseline, at the end of the study, or during the study. No significant change was found within groups during the study (Figure 7c).

4.4.8 Serum Free Fatty Acid (FFA) ALA
Among all the serum samples available (n=237), data from 3 subjects (1 from control and 2 from test) from week 4, 4 subjects (3 from control and 1 from test) from week 10 and 1 subject in the control group from 12 were missing due to the contamination of samples. In total, 96.624% samples were analyzed.

### 4.4.8.1 Percentage Composition of FFA ALA

No significant difference was observed at baseline between groups. FFA ALA for controls was 0.99±0.05%, and was 0.93±0.07% for test subjects. No significant change was found in controls during the intervention. During the study, FFA ALA was increased by 0.27% in the test group compared to its baseline (P= 0.004). The test group had an increase of 0.32% in serum FFA ALA compared with controls (P=0.002) (Figure 6d).

### 4.4.8.2 Concentrations of FFA ALA

Average FFA ALA concentration was not significant between groups at baseline: 2.75±0.24 μM for controls and 2.53±0.19 μM for test subjects at baseline. No significant difference was found between groups at baseline, at the end of the study, or during the study. No significant change was found within groups during the study (Figure 7d).

### 4.4.9 Relation of PSA change to Serum ALA change

#### 4.4.9.1 Percentage Composition

Total lipid ALA change and FFA ALA change were inversely associated with PSA change (r=-0.307, n=58, P=0.019; r=-0.356, n=56, P=0.007, respectively) (Figure 8a, 8b). However, the association became not significant after removal of outliers: 4 outliers in PSA, 2 outliers in total lipid ALA, and 1 outlier in FFA ALA. Phospholipid ALA change and triglyceride ALA change were not associated with PSA change in serum (r=-0.165, n=54, P=0.233; r=-0.246, n=56, P=0.069, respectively).
4.4.9.2 Concentrations

Serum triglyceride ALA concentration change was inversely correlated with change in PSA (r=-0.292, n=54, P=0.030) (Figure 9). However, after removal of 5 outliers (4 outliers in PSA and 1 outlier in triglyceride ALA), the result became not significant. Total lipid ALA, phospholipid ALA and FFA ALA were not associated with PSA change (r=-0.214, n=54, P=0.113; r=-0.118, n=54, P=0.400; r=-0.215, n=58, P=0.105, respectively).

4.5 Discussion

Consumption of an ALA-enriched diet (2.67g/day) for 3 months resulted in a decrease of 0.43 ng/ml in PSA compared to controls (P=0.014). Increased percentage composition of serum total lipid ALA as well as in its three lipid fractions (free fatty acids, phospholipids and triglycerides) was observed (P<0.05). Only concentrations of total lipid ALA and phospholipid ALA were increased in the test group compared with controls (P=0.015, P=0.035, respectively). Percentage composition changes in FFA ALA and total ALA, as well as change in triglyceride ALA concentration were associated with changes in PSA. However, the association was not significant after removal of outliers.

One intervention study also found an increase in percentage composition of ALA in serum after consuming ALA supplementation for 40 months. In the Alpha Omega Trial, patients in the ALA-arm who were provided with ALA-enriched margarine (2g ALA/d) experienced a 64.7% ALA increase in percentage composition after 20 months (P<0.001). The increase was sustained during the following study period, and the ALA-arm ended up with a 66.9% increase in ALA compared with placebo after 40 months (P<0.001)\cite{22}. Instead of measuring ALA in plasma, Azrad et al. randomized prostate cancer patients awaiting prostatectomy to receive a low fat diet, or a flaxseed supplemented diet (6.51g ALA/day), or both, and fatty acids in prostatic tissue were analyzed. They reported no significant difference between groups in ALA in prostatic tissue after dietary intervention for ~31 days\cite{95}.

Different from our PSA findings, both of the two studies described above reported a non-significant change in PSA after dietary intervention. ALA supplementation in the Alpha
Omega Trial resulted in an increase of PSA by 0.11 ng/ml (95%CI: -0.09, 0.32). The flaxseed supplementation study reported an increase of 0.7 ng/ml (P=0.056) while we observed a significant decrease of -0.438 ng/ml (P=0.040) in ALA arm when compared with control group. Intriguingly, a pilot study from the flaxseed supplementation study, which applied the same dietary intervention, but with 15 potential prostate cancer patients awaiting repeat biopsy, showed a 2.75 ng/ml decrease in PSA after 6-months intervention (P=0.0002)\textsuperscript{[24]}

The results revealed by the flaxseed supplementation study and its pilot study suggested that the effect of ALA might vary due to the progression of the disease. This was supported by another pilot study from the flaxseed supplementation study, which included 25 prostate cancer patients awaiting prostatectomy and used the same intervention as the flaxseed supplementation study. In that study, PSA was non-significantly increased by 0.36 ng/ml after \textasciidot34 days consuming flaxseed which was similar to the flaxseed supplementation study. However, when the results were broken down by Gleason score, patients with a Gleason score \geq 7 showed the opposite effect to patients with a Gleason score <7. While the former group experienced an increase in PSA, the later group showed a decrease although none of the results reached statistical significance\textsuperscript{[23]}. The Health Professional Follow-up Study suggested ALA was not related to increased prostate cancer risk when the results were restricted to organ-confined prostate cancer, but subjects with an ALA intake in the highest category had an almost two fold risk of advanced prostate cancer compared to those whose intakes were in the lowest quintile in the same study (RR=1.98, 95%CI: 1.34-2.93, p=0.001). Although some studies failed to find the correlation between ALA and advanced prostate cancer, none of the studies so far reported a negative association between ALA and advanced prostate cancer\textsuperscript{[47, 59, 90]}

The argument of whether lipids should be expressed on a percentage basis or a concentration basis is ongoing\textsuperscript{[155-158]}. Results analyzed by gas chromatography are always expressed as percentage composition (area normalization) in order to make adjustment for the difference in the total amount of fatty acids between subjects, and this method was used by most studies\textsuperscript{[12, 22, 92, 159]}. However, in this method, fatty acids were not independent and their values were narrowed by normalization\textsuperscript{[156, 157]}. Thus, we presented data on a concentration basis as well, and also to make it comparable to our later cell culture study. No significance within group change or between group changes was found during the study for FFA ALA and triglyceride ALA. The test
group had significantly higher phospholipid ALA and total lipid ALA during the study when compared to the controls (P=0.035, P=0.015, respectively).

One of the reasons that may account for the inconsistent results in the relation between dietary ALA intake and prostate cancer risk is differences in biomarkers chosen between studies. Our study showed that even within the same study, inclusion of different lipid fractions differentially affected the correlation between change in ALA and change in PSA. Changes in percentage composition of total lipid ALA and FFA ALA, but not phospholipid ALA and triglyceride ALA, were inversely associated with changes in PSA (r=-0.307, P=0.019; r=-0.356, P=0.007, respectively). However, after removal of outliers, change in none of the fraction was associated with change in PSA.

In our study, change in phospholipid ALA was not correlated with changes in PSA, which was not consistent with two other observational studies using the same lipid fraction[53, 92]. Yang et al. compared fatty acids profiles in serum of normal subjects, patients with benign prostatic hyperplasia and prostate cancer patients, and found that the percentage composition of ALA in serum fatty acids was the highest in prostate cancer patients, and lowest in normal patients (P for trend <0.05)[92]. A study in Norway reported an increased proportion of phospholipid ALA in prostate cancer cases compared with healthy subjects (P=0.02)[53].

We found a negative association between the percentage composition of total lipid ALA and FFA ALA and PSA, which was not reported by other observational studies[60, 61, 86]. A population-based cohort study reported that the percentage composition of total lipid ALA in serum was not related to the incidence of prostate cancer. Newcomer et al. suggested that those who were in the highest quartile of the percentage composition of total lipid ALA in erythrocytes had 2.6-fold increased prostate cancer risk compared to those in the lowest quartile (P=0.01) [61, 86]. Godley et al. measured free fatty acids in adipose tissue in both prostate cancer patients and healthy subjects free of cancer, and reported a non-significant increased risk of prostate cancer for those who were in the highest quartile compared with the lowest quartile[60]. However, the association became non-significant after removal of outliers.
4.6 Strengths and limitations

This study has strengths and limitations. First, this study was one of the few studies that looked at the effect of ALA on prostate cancer through a randomized-controlled trial. The within group and between group changes were the result of dietary intervention, which, compared with observational studies, was less prone to biases such as recall bias. Second, the extra 2.67g of ALA provided in our study as a supplement was higher than the minimum recommendation for ALA intake in Canada, and was within the most often proposed values (2 - 2.5g/day)[3]. The amount of ALA is realistic to reach from the diet as the highest quartile or quintile of ALA intake in other studies was above this amount[59]. Third, this study showed how serum lipid fractions changed (except cholesterol esters) after ALA consumption over a defined period of time, and established links between different serum lipid fractions and PSA to help to understand this gap in the literature. The use of diet histories and serum lipid analysis eliminated the possibility of recall bias from food frequency questionnaires, which was the limitation of most of the observational studies.

However, the results from the serum biomarker should be carefully interpreted since compared to observational studies, our study time was relatively short (12 weeks). Also, the cholesterol esters, which were mentioned by others as a better biomarker of ALA intake have not been analyzed at this time[3]. Meanwhile, the controversies over whether adipose tissue or erythrocytes should be a better biomarker for long-term intake, and whether weight percentage or absolute concentrations of fatty acids should be used to interpret data, also have to be taken into account. The second limitation of our study was that instead of using prostate cancer incidence, we used PSA as an indicator of risk of prostate cancer. The increase in PSA may not only relate to prostate cancer, but also to other prostatic diseases such as prostatitis and benign prostatic hyperplasia. Moreover, our study population was clinically free of cancer and had PSA values maller than 4 ng/ml at baseline. After intervention, PSA levels were mostly within the normal range (≤ 4 ng/ml), which might be difficult to translate to patients with clinically significant prostate cancer. Nevertheless, it is likely that our subjects had cancer cells in their prostate since with each advancing decade nodes of cancer cells were more common, with the incidence from 10% of men in the 50s to 70% of men in the 80s[160]. More intervention studies with prostate cancer patients with a longer study time should be carried out to fill the gap in our knowledge. Lastly, this study is a secondary
analysis and the original study was designed to explore the effect of canola oil on type 2 diabetes. The study bread was enriched with canola oil, which was high in ALA, but also a rich source of oleic acid. It is difficult to rule out the effect of oleic acid on the outcome. However, the difficulty of making substitutions was the same with other intervention studies that used manipulated foods such as the Alpha Omega Trial, which used ALA or EPA to replace oleic acid in the margarine[22]. We do know that patients with type 2 diabetes have a lower incidence of prostate cancer[134]. Thus the results from this study should be carefully interpreted before translating them to the subjects without diabetes.

4.7 Conclusion

This study suggested that supplementation of ALA for 12 weeks increased the percentage composition of ALA in all serum lipid fractions tested, but only increased ALA concentrations in total lipid ALA and phospholipid ALA. The intervention resulted in a reduction of PSA in the test group compared to controls over the time. Results from fatty acid analysis showed that only the percentage composition of total lipid ALA and FFA ALA, and the concentration of FFA ALA were significantly inversely associated with change in PSA. More studies are needed to be carried out to confirm these correlations. Since this study was a secondary analysis, and the PSA was mostly within the normal range in this study and more studies focusing on prostate cancer patients also need to be carried out.

<table>
<thead>
<tr>
<th>Table 1 - Nutrition Facts of Study Bread (per 500 cal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Bread</td>
</tr>
<tr>
<td>Energy (cal)</td>
</tr>
<tr>
<td>Moisture (g)</td>
</tr>
<tr>
<td>Protein (g)</td>
</tr>
<tr>
<td>Total Fat (g)</td>
</tr>
<tr>
<td>Saturated Fatty Acids (g)</td>
</tr>
<tr>
<td>Monounsaturated Fatty Acids (g)</td>
</tr>
<tr>
<td>Polyunsaturated Fatty Acids (g)</td>
</tr>
<tr>
<td>α-Linolenic Acid (g)</td>
</tr>
<tr>
<td>Linoleic Acid (g)</td>
</tr>
<tr>
<td>Total Carbohydrate (g)</td>
</tr>
<tr>
<td>Fiber (g)</td>
</tr>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Number of participants</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
</tr>
<tr>
<td>African</td>
</tr>
<tr>
<td>East Indian</td>
</tr>
<tr>
<td>European</td>
</tr>
<tr>
<td>Far Eastern</td>
</tr>
<tr>
<td>Other Whites/Caucasian</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Hispanic</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Waist (cm)</td>
</tr>
<tr>
<td>Waist/Height (cm/cm)</td>
</tr>
<tr>
<td>Current Smokers</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
</tr>
<tr>
<td>Anti-hyperglycemic medications</td>
</tr>
<tr>
<td>Metformin</td>
</tr>
<tr>
<td>Sulfonylurea</td>
</tr>
<tr>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>DPP-4 inhibitors</td>
</tr>
<tr>
<td>Meglitinides (non-sulfonylurea)</td>
</tr>
<tr>
<td>a-Glucosidase inhibitors</td>
</tr>
<tr>
<td>Injectable GLP-2 analogue (Victoza)</td>
</tr>
<tr>
<td>Dietary ALA intake (g/d)</td>
</tr>
</tbody>
</table>
Figure 5 - Flow of participants through the study
Figure 5 - The effect of diet on PSA

Figure 6a - Percentage composition of total lipid ALA during the study

Figure 6b - Percentage composition of phospholipid ALA during the study
Figure 6c - Percentage composition of triglyceride ALA during the study

Figure 6d - Percentage composition of FFA ALA during the study

Figure 7a - Total lipid ALA concentrations during the study
Figure 7b - Phospholipid ALA concentrations during the study

Figure 7c - Tryglyceride ALA concentrations during the study

Figure 7d - FFA ALA concentrations during the study
Figure 8a - Correlation of change in PSA and change in total lipid ALA (percentage composition) during the study (with outliers)

Figure 8b - Correlation of change in PSA and change in FFA ALA (percentage composition) during the study (with outliers)
Figure 9 - Correlation of change in PSA and change in triglyceride ALA (concentrations) during the study (with outliers)
Chapter 5
The Effect of ALA on Human Prostate Cancer Cells

5 The Effect of ALA on Human Prostate Cancer Cells

5.1 Abstract

**Background:** Although EPA and DHA have been reported to reduce prostate cancer risk, ALA, as the parent fatty acid of n-3 fatty acids, was reported to either positively or negatively affect prostate cancer in clinical trials. Results from in vitro studies have shown the inhibitory effect of ALA on prostate cancer cell growth. However, most of the doses used in cell culture studies were higher than the physiological range we measured in our previous study.

**Objectives:** To determine the effect of ALA on prostate cancer cell growth and on PSA production by LNCaP cells using the serum ALA concentrations analyzed in our previous study. To compare the effect of ALA with other C18:0 fatty acids.

**Methods:** LNCaP cells were plated at the same density and maintained in cell culture medium supplemented with 10% pooled human serum for 24 hours. The medium was then changed to treatment medium including standard cell culture medium supplemented with different concentrations of ALA, LA, oleic acid and stearic acid for 72 hours. Supernatant from the cells was used for PSA measurement by the AlphaLISA assay. Cell growth was measured by the MTS assay.

**Results:** No significant change in cell growth was observed for ALA at concentrations lower than 300 μM (μmol/L). 300 μM of ALA reduced cell growth by 25% (P<0.001). However, the same inhibitory effect was observed with other unsaturated C18 fatty acids. Thus, the reduced cell growth was mostly likely to be a fatty acid effect. No significant difference of PSA production was observed for ALA at concentrations lower than 200 μM. PSA production was suppressed at 200 μM and 300 μM of ALA (P=0.02, P<0.001, respectively), but the same reduction was observed with other C18 fatty acids as well.

**Conclusion:** We found no inhibitory effect of ALA on cell growth and PSA production by the LNCaP cells within the physiological concentrations of fasting blood.
5.2 Introduction

Alpha-linolenic (ALA) acid has been reported to be protective for coronary heart disease (CHD) by many studies\cite{9, 161, 162}. As the parent fatty acid of omega-3 fatty acids, ALA synthesizes eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by elongation and desaturation, and can further be metabolized to anti-inflammatory eicosanoids\cite{163}. The conversion of ALA to EPA in the body is 5-10%, and DHA levels are little influenced by ALA supplementation\cite{102}. ALA is postulated to suppress prostate cancer because of its competition with arachidonic acid derived pro-inflammatory eicosanoids for elongase, desaturase, cyclooxygenase and lipoxygenase during the eicosanoids synthesis\cite{82}. Increased EPA and DHA intakes were identified by many studies via inhibiting prostate tumor growth, reducing prostate cancer incidence as well as improving survival rate\cite{115, 35, 103}. However, results of increased ALA intake are inconsistent: some of the studies found a protective effect, while the others found it was associated with stimulating or a null association with prostate cancer\cite{9-11, 18-20, 53, 63, 84}. The majority of the studies are observational studies, the results of which varied by the population, other nutrients intake, food frequency questionnaire, follow-up duration, etc. These studies have not be linked to cell culture studies to provide relevant ALA concentrations in the physiological range and determine the effect of various concentrations of ALA on cancer cell growth.

Results from Prinsloo et al., Liu et al., and Eser et al. both showed that increased ALA concentration was negatively associated with prostate cancer cell growth\cite{35-37}. However, the majority of concentrations used in their studies were above 100 μM (μmol/L). According to the results from our previous study, the mean fasting serum ALA concentration was from 3 μM (free fatty acids) to 30 μM (total lipids), so if serum ALA reached 100 μM, it would likely to be after food intake. One study showed that ALA significantly increased cell death at 20 μM and 40 μM, but not at 4 μM, while another showed no significant difference at 10 μM\cite{37, 38}. More studies need to be done at lower concentrations to explore the effect of fasting ALA levels on human prostate cancer cells.

The purpose of the study was to explore the effect of ALA on prostate cancer cell growth and PSA produced by the cells using doses covering the fasting serum ALA concentrations from our
previous study. Other C18:0 fatty acids, including stearic acid (C18:0), oleic acid (C18:1 n-9),
and linoleic acid (C18:2 n-6) were also used in the study in order to compare with ALA.

5.3 Methods

5.3.1 Cell Line

The human prostate cancer cell line LNCaP was purchased from the American Type Culture
Collection (Rockville, Maryland, U.S.) and was grown in Roswell Park Memorial Institute
(RPMI) 1640 medium without phenol red (Thermo Fisher Scientific, Burlington, Ontario)
supplemented with 10% pooled human serum (PHS) (Labquip Ltd, Woodbridge, Ontario) in 37°C
humidified air containing 5% CO₂. Cells were subcultured when they reached ~80-90% 
confluence. The culture medium was changed every 2 days and cells were checked under the
microscope to make sure there was no contamination. All cell culture analysis was conducted
within a passage number of 10.

5.3.2 Subculture procedure

When cells reached ~80-90% confluence, they were washed by phosphate-buffered saline (PBS)
(Thermo Fisher Scientific, Burlington, Ontario), and then were treated with trypsin in the
incubator (37°C, 5%CO₂). Serum free RPMI 1640 was used to stop trypsin activity. The cell
culture mixture was centrifuged and cells were transferred to a new flask with standard culture
medium (10%PHS RPMI 1640 medium).

5.3.3 Cell Culture Plating and Treatment

LNCaP cells were seeded in each well of clear 96-well plates at a density of 5×10³ cells/well in
100 μl of 10%PHS RPMI medium. The outermost wells of the 96-well plate were not seeded and
contained only 10%PHS RPMI medium due to the evaporation reported by other researchers for
the following assays[164]. Cells were checked under the microscope and were allowed to grow in
the incubator (37°C, 5%CO₂). Twenty-four hours after plating, cells were checked again under
the microscope to make sure there was no contamination, no abnormalities, and cells were adhered to the bottom of the plate. Then 70 \( \mu l \) of the cell culture medium in each well was removed and replaced with 100 \( \mu l \) of treatment medium. For the outermost wells, 30 \( \mu l \) of 10\%PHS RPMI medium were added to keep the same volume as other wells. The cells of each well were checked under the microscope and were allowed to proliferate for 72 hours in the incubator (37\°C, 5\%CO\(_2\)). After 72 hours, cells were checked again and were used for assays.

5.3.4 Treatment medium

Stearic acid, oleic acid, linoleic acid and alpha-linolenic acid (Sigma-Aldrich, Toronto, Ontario) were dissolved in ethanol, and mixed with PHS to make 4 mM (mmol/L) stock solutions. Stock solutions were then diluted with different amount of PHS and RPMI 1640 medium to prepare the treatment medium of various concentrations. Taking into account that the volume of medium in each well was changed from 100\( \mu l \) to 130\( \mu l \) after replacement with treatment medium, the concentrations of the treatment medium were adjusted accordingly. After replacing with the treatment medium, the final concentrations for each fatty acid in the wells were 0 \( \mu M \) (control), 3 \( \mu M \), 5 \( \mu M \), 10 \( \mu M \), 30 \( \mu M \), 50 \( \mu M \), 100 \( \mu M \), 200 \( \mu M \) and 300 \( \mu M \). The final volume percentage of ethanol in which the fatty acid had been dissolved in each well was 0.42\%.

5.3.5 PSA Measurement

PSA produced by the cells was measured from 5 \( \mu l \) of supernatant in each well by PSA (human) AlphaLISA Detection Kit (Perkin-Elmer, Woodbridge, Ontario). AlphaLISA (Amplified Luminescent Proximity Homogeneous Assay). The PSA detection kit is a no-wash, no-separation homogenous assay kit for detection and quantitation of PSA in cell culture medium. Five micro mol of supernatant from each well was transferred to white 1/2 area 96 well plates (Perkin-Elmer, Woodbridge, Ontario) and mixed with AlphaLISA anti-analyte acceptor beads, biotinylated antibody anti-analyte and AlphaLISA immunoassay buffer to make a final concentration of 1 nM (nmol/L). Plates were incubated for 60 minutes at room temperature to allow antibodies and acceptor beads to bind with PSA. Then Streptavidin-coated donor beads and AlphaLISA immunoassay buffer mix was added to each well to make a final concentration of 40\( \mu g/ml \) in the dark. Plates were wrapped with aluminum foil and were incubated for 60
minutes at room temperature in the dark. Plates were read using EnSpire Multimode Plate Reader (Perkin-Elmer, Woodbridge, Ontario) at 615nm. Each concentration of stearic acid and oleic acid was replicated with 6 wells. Each concentration of ALA and LA was replicated with 12 wells. All results were expressed as a percentage of their control (0 μM).

5.3.6 Cell Proliferation Assay

The number of viable cells in the proliferation assay was measured by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI) using a colorimetric method. After measuring PSA, 100 μl of the remaining treatment medium in the 96-well plate was removed and replaced with 120 μl MTS mixture containing a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], an electron coupling reagent (phenazine ethosulfate; PES) and serum free RPMI 1640 medium (ratio of 1:5) were added in the dark. Plates were wrapped with aluminum foil and were incubated for 2 hours in the incubator (37°C, 5%CO₂). The serum-free medium volume in the outermost wells of the plates was adjusted to the same volume as the wells containing MTS dye. After incubation, plates were read with the absorbance of 490nm at 37°C by xMark™ Microplate Absorbance Spectrophotometer (Bio-rad, Berkeley, California). Each concentration of stearic acid and oleic acid was replicated with 6 wells. Each concentration of ALA and LA was replicated with 12 wells. All results were expressed as a percentage of their control (0 μM).

5.3.7 Statistical Analysis

Statistical analysis was performed by SAS 9.4. Data were presented as mean ± SEM. The statistical significance between doses of each fatty acid was found by a mixed-effects model, where both plate and position were treated as random effects and the Tukey adjustment used for multiple pairwise comparisons. The correlation between concentrations and PSA or cell growth, and the correlation between PSA and cell growth were assessed by Pearson's correlation. The probability level < 0.05 was considered significant.
5.4 Results

5.4.1 Descriptive Data from Clinical Trial

Table 3 showed the descriptive data of the concentrations of ALA in fasting serum from our clinical trial.

5.4.2 Cell Proliferation Assay (MTS)

5.4.2.1 Alpha-Linolenic Acid

No significant difference was found when ALA concentrations were 3 μM, 5 μM, 10 μM, 30 μM, 50 μM, 100 μM or 200 μM. LNCaP cell growth was significantly reduced by 25% at 300 μM compared to the 10% PHS control (P=0.001) (Figure 10a).

5.4.2.2 Linoleic Acid

LNCaP cell growth was not significantly different at 3 μM, 5 μM, 10 μM, 30 μM, 50 μM, 100 μM or 200 μM. LA inhibited cell growth by 34% at 300 μM (P<0.001) compared to the 10%PHS control (Figure 10b).

5.4.2.3 Oleic Acid

LNCaP cell growth was not significantly different at 3 μM, 5 μM, 10 μM, 30 μM, 50 μM or 100 μM. At 200μM and 300μM, LNCaP cell growth was suppressed by 17% and 56% (P<0.001, P<0.001, respectively) compared to the 10%PHS control (Figure 10c).

5.4.2.4 Stearic Acid
Compared to the controls, LNCaP cell growth was significantly inhibited by all the concentrations of stearic acid. No significant difference was observed between doses (Figure 10d).

5.4.2.5 C18 Fatty Acids

After combining the results of C18 fatty acids, we found no significant difference of cell growth at concentrations below 200 μM. Cell growth was inhibited by 12% at the concentration of 200 μM (P<0.01), and by 30% at 300 μM (P<0.001) (Figure 10e).

5.4.3 PSA Produced from the Cells

5.4.3.1 Alpha-Linolenic Acid

No significant difference of PSA production was observed at 3 μM, 5 μM, 10 μM, 30 μM, 50 μM or 100 μM. PSA production was suppressed at 200 μM by 22%, and reduced by 39% at 300 μM (P=0.02, P<0.001, respectively) (Figure 11a).

5.4.3.2 Linoleic Acid

PSA was not significantly different from the 10%PHS control at 3 μM, 5 μM, 10 μM, 30 μM, 50 μM or 100 μM. PSA started to be suppressed at 200 μM (P<0.001) and 300 μM (P<0.001) (Figure 11b).

5.4.3.3 Oleic Acid

PSA production was stimulated at 5 μM and 50 μM (P<0.01, P=0.047, respectively). And similarly, PSA decreased by 47% at 200 μM and 80% at 300 μM (P<0.001, P<0.0001, respectively) (Figure 11c).
5.4.3.4 Stearic Acid

PSA production was stimulated at 10 μM by 20% (P<0.01), and at 30 μM by 25% (P<0.01). No significant difference was found at other doses except a significant reduction was found at 300 μM (P<0.001) (Figure 11d).

5.4.3.5 C18 Fatty Acids

After combining the results of all the C18 fatty acids, we found that PSA production was significantly increased by 16% at 5 μM and 10 μM (P=0.004, P=0.003, respectively). The PSA production was suppressed at 200 μM and 300 μM by 32% and 42% (P<0.001, P<0.001, respectively) (Figure 10e).

5.4.4 Correlation of C18 fatty acids and Cell growth

By increasing C18 fatty acids from 3 μM to 300 μM, LNCaP cell growth was significantly negatively correlated with ALA (r = -0.882, n =9, P=0.002), LA (r = -0.953, n=9, P<0.0001) and OL (r = -0.940, n = 9, P<0.0001), but was not correlated with stearic acid. The negative correlation was largely due to a decrease at 200 μM and 300 μM (Figure 12a, 12b, 12c, 12d).

5.4.5 Correlation of C18 fatty acids and PSA

A reduction of PSA produced by LNCaP cells was observed as the concentration increased for ALA (r=-0.930, n=9, P=0.0002), LA (r=-0.973, n=9, P<0.0001), OL (r=-0.937, n=9, P=0.0002) and stearic acid (r=-0.833, n=9, P=0.005). The decreased was mostly due to a sharp drop at 200 μM and 300 μM (Figure 13a, 13b, 13c, 13d).

5.4.6 Correlation of Cell growth and PSA
Results showed that increase in cell growth also resulted in increase in PSA. There was a linear positive correlation between cell growth and PSA ($r=0.559$, $n= 274$, $P<0.001$) (Figure 14).

5.5 Discussion

Our study suggested no inhibitory effect of ALA on prostate cancer cell growth and PSA produced by the cells when the ALA was provided at the physiological concentration range of fasting blood.

ALA at concentrations lower than 300 $\mu$M did not show any significant difference of cell growth compared to 10%PHS controls, which was similar to the study of Liu et al. They maintained LNCaP cells in medium supplemented with 10% fetal bovine serum and ALA 1 $\mu$M, 100 $\mu$M or 500 $\mu$M. No statistical difference was found at 1 $\mu$M and 100 $\mu$M, while the cell growth was suppressed at 500 $\mu$M [37]. However, two other studies suggested a decreased cell growth at lower concentrations [35, 38]. Motaung et al. cultured DU-145 human prostate tumor cells in medium supplemented with decomplimented fetal calf serum and ALA at the concentrations of 4 $\mu$M, 20 $\mu$M or 40 $\mu$M for 19 hours. Results showed that the percentage of dead cells was not different from controls at the concentration of 4 $\mu$M, but was significantly increased at the concentrations of 20 $\mu$M and 40 $\mu$M compared with controls ($P<0.05$) [38]. Eser et al. grew PC-3 and LNCaP cells in a medium supplemented with 1% fetal bovine serum and 100 $\mu$M ALA for 3 days, and showed a significant decrease in cell proliferation compared to controls at the third day of treatment ($P<0.01$) [35].

Cell growth was reduced by 25% when ALA was added at 300 $\mu$M ($P=0.001$) for 72 hours. However, other unsaturated fatty acids including linoleic acid, oleic acid also showed significant reductions at the same concentration, suggesting that the reduction might be an effect of C18 unsaturated fatty acids rather than an individual fatty acid. Stearic acid significantly inhibited cell growth starting from a very low concentration (3 $\mu$M). However, as concentrations increased, the cell growth was not further inhibited, which is different from other unsaturated fatty acids. The range of mean concentrations of ALA in fasting serum samples was from 3 $\mu$M (free ALA) to 30 $\mu$M (total ALA), and the highest concentration of ALA detected from the study serum was from total lipids at the concentration of 160 $\mu$M (data not shown). Thus it is unlikely that ALA showed
an inhibitory effect within the fasting physiological concentration range. However, the fed state may be different and lead to much higher ALA than fasting ALA concentrations.

Two studies looked at the association between ALA intake and prostate cancer using the biomarker PSA. One clinical trial, the Alpha Omega Trial, showed an increased association. Study arm was supplemented with ALA enriched margarine (2g/day while we used 2.67g/day) for 40 months, and increased proportion of ALA was observed in the serum (concentrations not shown in the study), at the same time, an increase of 0.11 ng/ml PSA was found in study arm compared with controls at the end of the study, but the 95% confidence interval was wide and included both negative or null treatment effect. A pilot study of flaxseed supplementation showed a negative correlation between ALA intake and PSA. When participants were supplemented with 30g of flaxseed every day (equals to approximately 6.51g ALA/day) for 6 months, PSA level was reduced compared with baseline, but ALA level in serum was not measured in that study. These results demonstrated inconsistencies in the area of research.

We did not observe increased or decreased PSA production by the cells at concentrations lower than 200 \(\mu\)M. Our previous clinical trial showed that the difference of free fatty acid (FFA) ALA between tests and controls at the end of the study was smaller than 1 \(\mu\)M, and the difference of total lipid ALA between groups was approximately 9 \(\mu\)M at week 12 (Data not shown). In addition, the highest concentration of FFA ALA measured from our clinical trial was 103 \(\mu\)M. Thus, within the physiological range of FFA ALA in the fasting blood, ALA did not affect the production of PSA from cells. ALA significantly suppressed PSA production at concentrations of 200 \(\mu\)M and 300 \(\mu\)M by 32% and 39% compared to controls (P=0.02, P<0.001, respectively). However, this effect was most likely due to the effect of C18 fatty acids since linoleic acid, oleic acid and stearic acid exerted similar inhibitory effect on PSA at same concentrations.

We observed that the PSA produced by the cells seemed to follow such a pattern that, albeit the changes were not significant, the PSA started to increase at lower concentration and reached a peak at the concentration of 5 \(\mu\)M or 10 \(\mu\)M, then started to slowly decrease from 10 \(\mu\)M to 100 \(\mu\)M. After that, the PSA continued to drop but dramatically from 100 \(\mu\)M, where statistical significance was observed. Lastly we combined all the fatty acids together as C18 fatty acids to see if the effect was for C18 fatty acids. Results showed that at 5 \(\mu\)M and 10 \(\mu\)M, PSA
production was significantly increased compared to the 10% PHS controls (P=0.004, P=0.003, respectively), and a significant decrease was found at 200 μM and 300 μM (P<0.001, P<0.001, respectively). It is possible that the lack of significance we observed in ALA at the concentration of 5 μM and 10 μM was due to the lack of power, more replicates being needed. However, it should be recognized that the trend of increase at 5 μM and 10 μM was not specific to ALA, thus it was more likely to be an effect of C18 fatty acids.

5.6 Strengths and Limitations

Our study has some strengths and limitations. First, this study was designed to use ALA concentrations in the cell culture study based on ALA concentrations in fasting human blood from our clinical trial. Second, the different nutrient and immune profiles in cell culture may limit the generalization of cell culture findings. We therefore tried to fill in gap by substituting fetal bovine serum or fetal calf serum with pooled human serum. The validation of using pooled human serum was tested by others[165]. However, cell culture studies will not mimic the whole body's immune processes and metabolism. Also, the pooled human serum itself contained fatty acids, which might have affected the outcome. However, the amount and source of pooled human serum was the same in that of controls, and we expressed data as a percent relative to the controls. Third, we used a new approach to test PSA produced from the cells, which was the AlphaLISA assay. Compared to western blotting and ELISA, this no-wash assay eliminated the chance of loss of PSA during the washing procedure in other assays.

5.7 Conclusion

In this in vitro study, we did not observe an effect of ALA on LNCaP cell growth or on PSA production at low concentrations. Although at high concentrations, ALA resulted in significantly inhibited cell growth and PSA production, the effect was the same with other unsaturated fatty acids and the concentrations that showed significant reduction were not at the physiological range of FFA ALA concentrations of fasting blood.
### Table 3 - Descriptive Data of Fatty Acids in Serum Samples ($\mu$M)

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
<th>Highest</th>
<th>Lowest</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>ALA 37.53±2.23</td>
<td>31.33±1.38</td>
<td>159.85</td>
<td>3.12</td>
</tr>
<tr>
<td></td>
<td>LA 915.62±28.59</td>
<td>972.07±31.18</td>
<td>1959.76</td>
<td>175.32</td>
</tr>
<tr>
<td></td>
<td>OL 962.38±32.29</td>
<td>921.71±35.49</td>
<td>2076.90</td>
<td>227.17</td>
</tr>
<tr>
<td></td>
<td>ST 285.81±6.35</td>
<td>281.03±6.07</td>
<td>625.55</td>
<td>135.01</td>
</tr>
<tr>
<td>FFA</td>
<td>ALA 2.78±0.12</td>
<td>2.72±0.15</td>
<td>103.33</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>LA 28.50±0.98</td>
<td>32.07±1.50</td>
<td>93.12</td>
<td>5.64</td>
</tr>
<tr>
<td></td>
<td>OL 87.51±3.03</td>
<td>93.79±3.70</td>
<td>244.76</td>
<td>23.16</td>
</tr>
<tr>
<td></td>
<td>ST 34.29±0.84</td>
<td>35.11±0.89</td>
<td>75.78</td>
<td>13.81</td>
</tr>
<tr>
<td>PL</td>
<td>ALA 3.05±0.16</td>
<td>2.77±0.25</td>
<td>11.41</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>LA 237.99±6.98</td>
<td>258.06±7.44</td>
<td>557.70</td>
<td>95.40</td>
</tr>
<tr>
<td></td>
<td>OL 140.72±3.32</td>
<td>142.81±3.19</td>
<td>290.65</td>
<td>59.63</td>
</tr>
<tr>
<td></td>
<td>ST 227.30±5.01</td>
<td>222.63±4.09</td>
<td>447.13</td>
<td>116.46</td>
</tr>
<tr>
<td>TG</td>
<td>ALA 8.62±0.78</td>
<td>6.83±0.48</td>
<td>42.62</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>LA 96.87±6.06</td>
<td>93.61±5.36</td>
<td>323.37</td>
<td>7.36</td>
</tr>
<tr>
<td></td>
<td>OL 266.04±14.65</td>
<td>246.89±14.66</td>
<td>793.84</td>
<td>29.63</td>
</tr>
<tr>
<td></td>
<td>ST 37.22±2.45</td>
<td>36.99±3.56</td>
<td>217.29</td>
<td>8.20</td>
</tr>
</tbody>
</table>

**Figure 10a** - Effect of different ALA concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
Figure 10b - Effect of different LA concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 10c - Effect of different oleic acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
**Figure 10d** - Effect of different stearic acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

**Figure 10e** - Effect of different C18 fatty acid concentrations on LNCaP cell growth. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
**Figure 11a** - Effect of different ALA concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

**Figure 11b** - Effect of different LA concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
Figure 11c - Effect of different oleic acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 11d - Effect of different stearic acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).
Figure 11e - Effect of different C18 fatty acid concentrations on PSA produced from the cells. Results are expressed as % compared with 10%PHS controls. Treatment means with different letters are statistically significant (P<0.05).

Figure 12a - Correlation of LNCaP cell growth and ALA concentrations
Figure 12b - Correlation of LNCaP cell growth and LA concentrations

Figure 12c - Correlation of LNCaP cell growth and oleic acid concentrations
Figure 12d - Correlation of LNCaP cell growth and stearic acid concentrations

Figure 13a - Correlation of PSA produced from the cells and ALA concentrations
Figure 13b - Correlation of PSA produced from the cells and LA concentrations

Figure 13c - Correlation of PSA produced from the cells and oleic acid concentrations
Figure 13d - Correlation of PSA produced from the cells and stearic acid concentrations

Figure 14 - Correlation of LNCaP cell growth and PSA produced from the cells
Chapter 6
Overall Discussion

6 Overall Discussion

6.1 Discussion

Prostate cancer is the third most common cancer worldwide\textsuperscript{[3]}. The cost for medical treatment is over $11 billion a year in the U.S. alone and the number is predicted to increase in the following years\textsuperscript{[2]}. In Canada, it contributes to around 25% of new cancer cases and 10% of cancer death each year\textsuperscript{[1]}. As the parent fatty acid of the n-3 polyunsaturated fatty acids family, ALA has been reported to decrease coronary heart disease incidence and mortality but the reverse association has been suggested for prostate cancer.

Our study used data and serum samples from a randomized controlled study and observed that ALA supplementation significantly increased the proportion of ALA in serum total lipids, phospholipids, triglycerides and free fatty acids. The serum ALA concentration did not change significantly in total lipids, phospholipids and triglycerides but was significantly increased in free fatty acids during the intervention. The extra 2.67g of dietary ALA significantly lowered PSA in the test group by -0.438 ng/ml compared to controls during the study. Our study also revealed that different serum lipid fractions differentially affected the PSA. The proportion of free ALA and total ALA was inversely correlated with PSA while no correlation was detected in phospholipid ALA and triglyceride ALA.

We further conducted an in vitro study on the basis of serum ALA concentrations obtained from the randomized controlled study. No significant change in prostate cancer cell growth was observed in doses lower than 300\textmu M ALA. At 300\textmu M, ALA significantly decreased prostate cancer cell growth but the effect was more likely to be a general fatty acid effect since a similar inhibition was found with the other C18:0 fatty acids. Similarly, PSA production was not inhibited at low concentrations, but was suppressed at 200 \textmu M and 300 \textmu M, similar to other C18:0 fatty acids.
6.2 Future Work

Plasma cholesterol esters are important biomarkers for long-term dietary intake, and constituted the largest portion of lipid fraction in our study[3]. In a following analysis, cholesterol esters from our clinical trials will be analyzed with the same method as other lipid fractions in order to fill this gap in our fatty acid profile.

Although analyzing serum fatty acids concentrations reduced the dependence of food frequency questionnaires with possible recall bias which was common in most other studies, there is no consensus on which biomarker is more sensitive and can best represent changes in diet. In the future, dietary intake of ALA from our clinical trial will be extracted from the food frequency questionnaires obtained during the study, and changes in dietary ALA intake will be calculated and be used to compare with the ALA measures from the serum and serum PSA.

Androgens, which are important sex hormones for the development of the normal prostate gland, also play an important role in the development and progression of prostate cancer[166]. The suppression of androgens by using androgen deprivation therapy (ADT) has proved to be effective in controlling prostate cancer growth in its early stages[167, 168]. Unfortunately, patients will develop chemical castration-resistant prostate cancer, the survival rate of which is only 16-18 months after 2-3 years of ADT, so new alternative treatments are required to control sex hormones and their receptors[169]. In the future, sex hormones levels from participants' serum will be measured to explore the effect of ALA.

The development and the normal function of the prostate also depends on the androgen receptor, which is a transcription factor that binds to testosterone and 5α-dihydrotestosterone (DHT)[170]. Androgen receptor activity is also closely related to prostate cancer. Increased androgen receptor activity is always observed in prostate cancer patients[171]. In future cell culture studies, modification of androgen receptor activities by diet components will be interesting to investigate.
Besides our study, flaxseed supplementation studies and the Alpha Omega Trial, there are few intervention studies available in this area. However, even our study and the study from Alpha Omega Trial were both secondary analysis from randomized control trials, and the outcomes from the original studies were unrelated to prostate cancer[^22]. The objectives of the flaxseed supplementation studies were to look at the effect of fat restriction and flaxseed supplementation (contains lignans) on prostate cancer, and the follow-up time was comparatively short (~31 days[^23-25, 95]). In the future, long-term intervention studies focused on ALA supplementation in prostate cancer patients are needed.

Our study failed to find significant reduction of the growth of prostate cancer cells and PSA production from the cells within physiological ranges of human fasting serum ALA concentrations. However, our fasting values for ALA may be lower, and day long exposure is probably more important. Therefore the day profile of ALA is required including postprandial values. In the future, more cell culture studies are needed to look at the effect of ALA using clinical trial based concentrations instead of doses that are far from physiological ranges.
Chapter 7
Conclusion

7 Conclusion

1. ALA supplementation resulted in an increased percentage composition of ALA in total lipids, free fatty acids, phospholipids and triglycerides, and decreased PSA.

2. No inhibitory effect on prostate cancer cell growth and PSA produced from the cells was observed when ALA was provided at physiological concentrations.

3. The effect of ALA is still in question. More randomized controlled trials and more cell culture studies using physiological concentrations need to be conducted.
References

34. Sasaki, M., et al., Low percentage of free prostate-specific antigen (PSA) is a strong predictor of later detection of prostate cancer among Japanese men with serum levels of total PSA of 4.0 ng/mL or less. Urology, 2014. 84(5): p. 1163-7.
86. Laaksonen, D.E., et al., *Serum linoleic and total polyunsaturated fatty acids in relation to
106. Kobayashi, N., et al., Effect of altering dietary omega-6/omega-3 fatty acid ratios on


121. Li, H., et al., Tumor cell group via phospholipase A(2) is involved in prostate cancer development. Prostate, 2011. 71(4): p. 373-84.


123. Wilt, T.J. and P. Dahm, PSA Screening for Prostate Cancer: Why Saying No is a


# Appendices

## Appendix 1 - Effect of Diet on Percentage Composition of Linoleic Acid

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 10</th>
<th>Week 12</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26.08±0.62</td>
<td>26.15±0.81</td>
<td>24.34±0.66</td>
<td>25.99±0.88</td>
<td>0.29</td>
</tr>
<tr>
<td>Test</td>
<td>24.09±0.78</td>
<td>24.95±0.87</td>
<td>26.02±0.78</td>
<td>24.91±0.97</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.002*</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.46±0.46</td>
<td>18.07±0.53</td>
<td>17.22±0.44</td>
<td>17.90±0.57</td>
<td>0.172</td>
</tr>
<tr>
<td>Test</td>
<td>17.18±0.53</td>
<td>17.09±0.60</td>
<td>17.87±0.58</td>
<td>18.07±0.53</td>
<td>0.033*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.016*</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.50±0.69</td>
<td>14.10±0.78</td>
<td>13.97±0.71</td>
<td>13.90±0.65</td>
<td>0.121</td>
</tr>
<tr>
<td>Test</td>
<td>12.69±0.79</td>
<td>13.15±0.60</td>
<td>13.59±0.83</td>
<td>13.15±0.92</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.833</td>
</tr>
<tr>
<td><strong>Free Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.30±0.43</td>
<td>11.64±0.55</td>
<td>10.61±0.45</td>
<td>11.46±0.46</td>
<td>0.54</td>
</tr>
<tr>
<td>Test</td>
<td>10.41±0.40</td>
<td>10.84±0.44</td>
<td>10.41±0.48</td>
<td>11.77±0.42</td>
<td>0.049*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.063</td>
</tr>
</tbody>
</table>

*P<0.05

## Appendix 2 - Effect of Diet on Percentage Composition of Oleic Acid

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 10</th>
<th>Week 12</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>23.91±0.53</td>
<td>23.34±0.76</td>
<td>24.32±0.78</td>
<td>23.43±0.68</td>
<td>0.508</td>
</tr>
<tr>
<td>Test</td>
<td>24.66±0.54</td>
<td>25.44±0.64</td>
<td>26.01±0.63</td>
<td>24.31±0.81</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.062</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.17±0.20</td>
<td>9.86±0.18</td>
<td>10.34±0.26</td>
<td>9.83±0.24</td>
<td>0.036*</td>
</tr>
<tr>
<td>Test</td>
<td>10.17±0.16</td>
<td>10.49±0.20</td>
<td>10.55±0.24</td>
<td>10.45±0.17</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.005*</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35.31±0.89</td>
<td>34.84±0.85</td>
<td>36.99±0.95</td>
<td>35.39±1.03</td>
<td>0.103</td>
</tr>
<tr>
<td>Test</td>
<td>33.78±0.81</td>
<td>37.18±0.76</td>
<td>38.19±0.93</td>
<td>35.86±1.77</td>
<td>0.007*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.293</td>
</tr>
<tr>
<td><strong>Free Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>33.06±0.60</td>
<td>33.80±0.67</td>
<td>32.67±0.79</td>
<td>33.25±0.78</td>
<td>0.528</td>
</tr>
<tr>
<td>Test</td>
<td>31.23±0.63</td>
<td>33.84±0.61</td>
<td>32.59±0.99</td>
<td>34.16±0.72</td>
<td>0.008*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.085</td>
</tr>
</tbody>
</table>

*P<0.05
### Appendix 3 - Effect of Diet on Percentage Composition of EPA

<table>
<thead>
<tr>
<th></th>
<th>Total Lipids</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
<th>Free Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td><strong>Total Lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.06±0.10</td>
<td>0.97±0.13</td>
<td>1.31±0.12</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td></td>
<td>1.14±0.15</td>
<td>1.06±0.14</td>
<td>1.36±0.17</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td></td>
<td>0.96±0.08</td>
<td>1.00±0.13</td>
<td>1.29±0.11</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td></td>
<td>1.03±0.08</td>
<td>1.05±0.12</td>
<td>1.25±0.11</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td></td>
<td>0.216</td>
<td>0.458</td>
<td>0.342</td>
<td>0.824</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25±0.01</td>
<td>1.25±0.01</td>
<td>1.39±0.17</td>
<td>0.28±0.06</td>
</tr>
<tr>
<td></td>
<td>1.39±0.17</td>
<td>1.39±0.17</td>
<td>1.21±0.12</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td></td>
<td>1.21±0.12</td>
<td>1.21±0.12</td>
<td>1.36±0.13</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td></td>
<td>1.36±0.13</td>
<td>1.36±0.13</td>
<td>0.456</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>0.233</td>
<td>0.313</td>
<td>0.456</td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.27±0.03</td>
<td>0.24±0.03</td>
<td>0.27±0.03</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td></td>
<td>0.27±0.02</td>
<td>0.28±0.06</td>
<td>0.27±0.02</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td></td>
<td>0.28±0.04</td>
<td>0.19±0.02</td>
<td>0.28±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.824</td>
<td>0.161</td>
<td>0.313</td>
<td></td>
</tr>
<tr>
<td><strong>Free Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td></td>
<td>0.10±0.01</td>
<td>0.08±0.01</td>
<td>0.10±0.01</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td></td>
<td>0.08±0.01</td>
<td>0.08±0.00</td>
<td>0.10±0.01</td>
<td>0.08±0.00</td>
</tr>
<tr>
<td></td>
<td>0.266</td>
<td>0.118</td>
<td>0.118</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>0.063</td>
<td>0.063</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05