Differential effects of long chain omega-3 polyunsaturated fatty acids on platelet aggregation and hemostatic variables in healthy male versus female subjects

Melinda Phang
(BSc)
School of Biomedical Sciences & Pharmacy

This thesis is presented for the Degree of Doctor of Philosophy
The University of Newcastle, Australia

November 2012
Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository subject to the provisions of the Copyright Act 1968.

Thesis by publication

Acknowledgement of Authorship

I hereby certify that the work embodied in this thesis contains published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty of Health Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Melinda Phang
Acknowledgments

Firstly, I would like to acknowledge my primary supervisor Manohar Garg, who has contributed significantly to my development through his guidance, encouragement and support. His intellect and positive outlook has given me great inspiration to continue my research trajectory and to overcome the many challenges of scientific research. I would also like to thank my co-supervisor, Lisa Lincz for her patience, invaluable feedback and passionate mentoring. She is a remarkable role model that has inspired me with her knowledge and guidance.

Thank you to the Hunter Medical Research Institute to enable my research studies in the capacity of participant recruitment. I am also thankful for all the wonderful and dedicated volunteers that I have encountered along the way. Thank you to EPAX for providing the capsules for my studies and to the National Health & Medical Research Council for providing me with my postgraduate scholarship that has allowed me to complete my PhD.

A most sincere thank you to my friends: Irene, Jency and Melissa in the Nutraceuticals Research Group. You have made this great crusade one that is filled with laughter, happiness and plenty of fond memories. Above all, one that is supportive and invaluable. Thank you to Fiona for your assistance and kind support in making my experience in the lab an enjoyable one. I would also like to thank my lovely and most dear friend Kylie Chan that has always been there for me. I will always be appreciative for her generous and caring nature as a true friend over the last ten years. It is friendship like these that create lasting memories and lifelong bonds.

Most importantly, I am extremely grateful to be blessed with such caring, loving and amazing parents. Thank you to my mum and dad for always believing in me and encouraging me to be the best that I can be. Mum, you have been my rock, providing me with your voice of reason and passionate upbringing. Lastly, thank you to my sister Melissa, and my brother Kieren for being there for me (and putting up with my tantrums) every step of the way. Sis, you have given me guidance though your love
and encouragement and you are truly the best big sister I could ever ask for. Words cannot explain how lucky I am to have each of you in my life.

I would like to end with a motivational quote to all graduate students when faced with the challenges of research.

“To accomplish great things we must not only act, but also dream, not only plan, but also believe.” - Anatole France
Publications and presentations arising from this thesis

Refereed Journal publications:


7. **Phang, M.**, Scorgie, F.E, Seldon, M., Garg, M.L & Lincz, L; ‘Reduction of prothrombin and Factor V levels following supplementation with omega-3 fatty
acids is gender-dependant: a randomised controlled study’ (Under review).
Submitted for publication in ‘Thrombosis Research’ on November 19 2012

Conference abstracts: published in peer-reviewed journals


Conference abstracts: published in conference proceedings

1. M. Phang, M.L. Garg; ‘Inhibition of platelet aggregation by LC n-3PUFA is gender specific’ 14th World Congress on Clinical Nutrition and 5th International Congress on Cardiovascular Diseases (4-7 June 2009) Kosice, Slovakia.


# Table of Contents

Table of Figures ........................................................................................................ 155
Table of Tables ........................................................................................................ 16
Abbreviations ............................................................................................................. 17

**Synopsis** ................................................................................................................ 19

**Thesis structure and overview** ................................................................................. 22

**Chapter One: Introduction and literature review** ..................................................... 26

1.1. *Background and context* ..................................................................................... 27

1.1.1. Cardiovascular and thrombotic disease ......................................................... 27

1.1.2. Platelet discovery .......................................................................................... 28

1.1.3. Platelet biology ............................................................................................. 29

1.1.4. Platelet lipid membrane ............................................................................... 29

1.1.5. Platelet surface .............................................................................................. 30

1.1.6. Platelet receptors ........................................................................................ 30

1.1.7. Platelet granules .......................................................................................... 32

1.1.8. Primary hemostasis ...................................................................................... 33

1.1.8.1. Platelet adhesion .................................................................................... 35

1.1.8.2. Platelet activation ................................................................................... 35

1.1.8.3. Platelet aggregation ............................................................................... 36

1.1.9. Secondary hemostasis .................................................................................. 36

1.1.9.1. Coagulation ............................................................................................ 36

1.1.9.2. Platelet procoagulant activity................................................................. 38

1.1.9.3. Membrane phospholipids ..................................................................... 38

1.1.9.4. Platelet-derived microparticles .............................................................. 39

1.1.10. Fibrinolysis .................................................................................................. 40

1.1.11. Principal physiological agonists of platelet activation and aggregation ........ 42

1.1.11.1. Collagen ................................................................................................ 42

1.1.11.2. Adenosine diphosphate ...................................................................... 42

1.1.11.3. Thrombin ............................................................................................... 43

1.1.11.4. Thromboxane A₂ .................................................................................. 43

1.1.11.5. Platelet procoagulant activity/platelet-derived microparticles .............. 43

1.1.11.6. Soluble P-selectin .................................................................................. 44

1.1.12. Hemostasis versus thrombosis: Maintaining the balance ............................ 44

1.1.13. Thrombosis and other CVD risk factors ....................................................... 45
1.1.14. Pharmacological anti-platelet agents: clinical outcomes and consequences......46

1.2. Long chain omega-3 polyunsaturated fatty acids: A non-pharmacological approach.................................................................49

1.2.1. Lipid structure and classification.................................................49

1.2.2. Dietary fatty acids.........................................................................49

1.2.3. Omega-3 fatty acid structure and metabolism..............................50

1.2.4. Ratios and recommendations........................................................55

1.2.3. Dietary fatty acids in cell membranes.............................................55

1.2.6. Eicosanoid formation ..................................................................56

1.2.7. Epidemiological, observational and prospective studies................58

1.2.8. The problem: a controversy..........................................................63

1.2.9. The Resolution: a controlled approach.........................................64

1.3. Cardiovascular gender differences in platelet aggregation: The possible underlying issue..............................................................66

1.3.1. Gender differences in thrombosis..................................................66

1.3.2. Cardiovascular and hemostatic gender differences........................67

1.3.3. Gender differences in LCn-3PUFA composition and metabolism....69

1.3.4. Conflict: EPA and DHA on platelet activity and coagulation...........71

1.3.4.1. Eicosanoid modification, TXB and bleeding time.........................76

1.3.4.2. Procoagulant activity and coagulation factors..............................81

1.3.4.3. PAI-1 and fibrinolysis.................................................................83

1.4. Gender-based nutraceutical approach: A possible solution................85

1.5. Research hypothesis and objectives.................................................86

1.5.1. Original hypothesis........................................................................86

1.5.2. Preliminary novel findings.............................................................87

1.5.3. Updated hypothesis: a new perspective..........................................87

1.5.4. Objectives.....................................................................................88

1.5.5. Research significance and anticipates outcomes............................89

Chapter Two: Methods..........................................................................92

2.1. Research study design......................................................................91

2.2. Participants.....................................................................................91

2.2.1. Study 1.......................................................................................91

2.2.2. Study 2.......................................................................................92

2.2.3. Study 3.......................................................................................92

2.3. Whole blood platelet aggregation.....................................................93

2.3.1. Study 1.......................................................................................94
2.2.2. Studies 2 and 3.................................................................94
2.4. Manual platelet count and blood biochemistry.............................95
2.5. Full blood count and sex hormonal levels..................................96
2.6. Measurement of TXB$_2$, vWF, MP activity, P-sel and PAI-1...............96
2.7. Determination of coagulation factor activity................................97
2.8. Endogenous Thrombin Potential..............................................98
2.9. Flow cytometry.......................................................................98
2.10. Plasma fatty acid analyses....................................................99
2.11. Anthropodmetry and Food Intake..........................................99
2.12. Statistical analyses..............................................................100

Chapter Three: Investigation of differential effects of individual LCn-3PUFA on platelet aggregation in vitro in human subjects..............101

3.1. Objective 1...........................................................................102
  3.1.1. Abstract.............................................................................102
  3.1.2. Introduction........................................................................102
  3.1.3. Study Design and Methods................................................104
    3.1.3.1. Participants...................................................................104
    3.1.3.2. Platelet function assays..............................................105
    3.1.3.3. Statistical analyses....................................................105
  3.1.4. Results...............................................................................106
  3.1.5. Discussion..........................................................................110

Chapter Four: Acute dietary supplementation with EPA and DHA rich oils on ex vivo platelet aggregation in male and female subjects......114

4.1. Objective 2...........................................................................115
  4.1.1. Abstract.............................................................................115
  4.1.2. Introduction........................................................................115
  4.1.3. Study Design and Methods................................................116
    4.1.3.1. Participants...................................................................116
    4.1.3.2. Platelet aggregation assays............................................117
    4.1.3.3. Blood analyses............................................................118
    4.1.3.4. Statistical analyses....................................................118
  4.1.4. Results...............................................................................118
    4.1.4.1. Baseline demographics...............................................118
    4.1.4.2. Platelet aggregation.....................................................120
  4.1.5. Discussion..........................................................................122

4.2. Objective 3...........................................................................126
5.2.1. Abstract........................................................................................................161
5.2.2. Introduction................................................................................................162
5.2.3. Study Design and Methods.......................................................................163
  5.2.3.1. Participants..............................................................................................163
  5.2.3.2. Blood analysis.........................................................................................164
  5.2.3.3. Platelet aggregation assays.................................................................164
  5.2.3.4. Determination of coagulation factor activity......................................165
  5.2.3.5. Endogenous thrombin potential.........................................................165
  5.2.3.6. Plasma fatty acid analyses.................................................................166
  5.2.3.7. Statistical analysis................................................................................166
5.2.4. Results........................................................................................................167
  5.2.4.1. Baseline demographics.......................................................................167
  5.2.4.2. Overall effects of supplementation..................................................169
  5.2.4.3. Gender effects of supplementation..................................................172
  5.2.4.4. Relationship between hormonal status, platelet aggregation, procoagulant activity and plasma fatty acid composition in response to supplementation..................................................172
5.2.5. Discussion................................................................................................175

Chapter Six: General Discussion.......................................................................179
  6.1. Key findings.................................................................................................180
  6.2. Research strength and limitations............................................................185
  6.3. Implications of the body of research.......................................................187
  6.4. Final conclusion.........................................................................................188

References..........................................................................................................189

Appendices..........................................................................................................233
  Appendix 1: Study 1 Information Statement & Consent Form..........................234
  Appendix 2: Study 2 Information Statement & Consent Form..........................238
  Appendix 3: Study 3 Information Statement & Consent Form..........................242
  Appendix 4: Study 1 & 2 Participant Assessment Criteria..................................246
  Appendix 5: Study 2 & 3 Participant Assessment Criteria..................................247
  Appendix 6: Pre-trial Medical Questionnaire.................................................248
  Appendix 7: 24 Hour Food Recall Form .........................................................250
  Appendix 8: Statement of Contribution for Chapter One..................................252
  Appendix 9: Statement of Contribution for Chapter One..................................253
  Appendix 10: Statement of Contribution for Chapter Three...........................254
  Appendix 11: Statement of Contribution for Chapter Four.............................255
Appendix 12: Statement of Contribution for Chapter Four ......................... 256
Appendix 13: Statement of Contribution for Chapter Five .......................... 257
Appendix 14: Statement of Contribution for Chapter Five .......................... 258
Table of Figures

| Figure 1-1 | Platelets in primary and secondary hemostasis | 34 |
| Figure 1-2 | Traditional model of the coagulation cascade | 37 |
| Figure 1-3 | Revised model of the coagulation cascade | 38 |
| Figure 1-4 | Generation of microparticles and their procoagulant activity | 40 |
| Figure 1-5 | The fibrinolytic system | 41 |
| Figure 1-6 | Chemical structure of n-3 and n-6 polyunsaturated fatty acids | 52 |
| Figure 1-7 | Synthesis of n-3 and n-6 polyunsaturated fatty acids to their longer chain derivatives | 54 |
| Figure 2-1 | Example of platelet aggregation curve | 95 |
| Figure 3-1 | Inhibitory effects of n-3PUFA on platelet aggregation in males and females combined | 107 |
| Figure 3-2 | Gender differences in response to LCn-3PUFA in platelet aggregation | 109 |
| Figure 3-3 | Relationship between baseline aggregation and platelet count in males and females combined | 109 |
| Figure 3-4 | Gender differences in relationship between baseline aggregation and platelet count in males and females | 110 |
| Figure 4-1 | Effect of supplementation on platelet aggregation over time in males and females | 121 |
| Figure 4-2 | Effect of supplementation on platelet aggregation over time in males | 121 |
| Figure 4-3 | Effect of supplementation on platelet aggregation over time in female | 122 |
| Figure 4-4 | Gender difference in the relationship between the baseline aggregation and MP activity | 134 |
| Figure 4-5 | Effect of LCn-3PUFA on platelet aggregation in males and females | 135 |
| Figure 4-6 | Effect of LCn-3PUFA on MP activity in males and females | 136 |
| Figure 5-1 | Effect of treatment and sex on platelet aggregation following 4 weeks supplementation | 154 |
| Figure 5-2 | Comparative changes in plasma fatty acids following EPA and DHA supplementation in males and females | 156 |
| Figure 5-3 | Differential effects of supplementation on hemostatic variables and platelet aggregation in males and females combined, male, and female cohort | 169 |
Table of Tables

Table 1-1. Platelet plasma membrane receptors.................................................................31
Table 1-2. Platelet α-granule contents in hemostasis.......................................................32
Table 1-3. LCn-3PUFA on platelet and hemostatic function............................................72
Table 3-1 Characteristics of the study participants.........................................................107
Table 4-1. Characteristics of study participants.................................................................119
Table 4-2. Correlations between characteristics and platelet aggregatory response........119
Table 4-3. Characteristics of study participants.................................................................132
Table 4-4. Correlations between platelet and MP parameters in the total cohort.............133
Table 5-1. Characteristics of study participants.................................................................133
Table 5-2. Correlations between hormonal status and platelet aggregatory response.....151
Table 5-3. Changes in hemostatic markers, platelet aggregation and plasma fatty acids
      post supplementation..................................................................................................152
Table 5-4. Differential changes in hemostatic markers, platelet aggregation and plasma
      fatty acids post supplementation in males and females...........................................153
Table 5-5. Characteristics of study participants.................................................................168
Table 5-6. Change in coagulation profile, hemostatic parameters and plasma fatty acids
      following dietary supplementation with EPA or DHA rich oils..............................171
Table 5-7. Differential effects of EPA or DHA rich oil supplementation on coagulation
      profiles and plasma fatty acids in males and female..............................................174
Table 5-8. Correlations between hormonal status, coagulation activity and platelet
      aggregatory response following 4 weeks supplementation......................................175
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linoleic acid</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CAM</td>
<td>Cellular adhesion molecule</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>Diaglycerol</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic acid</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenic acid</td>
</tr>
<tr>
<td>ETP</td>
<td>Endogenous thrombin potential</td>
</tr>
<tr>
<td>F</td>
<td>Factor</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>Fb</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>FO</td>
<td>Fish oil</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled transmembrane receptors</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic heart disease</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acids</td>
</tr>
<tr>
<td>LCn-3PUFA</td>
<td>Long Chain omega-3 polyunsaturated fatty acids</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipoxigenase</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeated receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>n-3</td>
<td>Omega-3</td>
</tr>
<tr>
<td>n-6</td>
<td>Omega-6</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease activated receptor</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>PCK</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PGI</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl inositol</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>PLG</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>P-sel</td>
<td>P-selectin</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
</tbody>
</table>
Synopsis

Thrombosis is a critical event that accounts for considerable morbidity and mortality in the Western world. Thrombosis is associated with arterial diseases including, myocardial infarction, stroke, and peripheral occlusive disease as well as with venous thromboembolic disorders. Consequently, the primary goal for the prevention of arterial and venous thrombosis to combat disease progression is to limit thrombus extension. Platelet activation and aggregation is considered to be central to thrombus production; thus anti-thrombotic treatments to inhibit platelet activity have been a major drug target to retard the thrombotic and atherosclerotic processes. Despite extensive resource investment in cardiovascular research and treatment, the current pharmacological strategies for the inhibition of platelet aggregation, although effective, may present limitations and adverse health effects have been reported. Given the toll taken by thrombotic complications, a safe and efficacious non-pharmacological approach may be paramount for the prevention and management of thrombotic disease.

While a wealth of evidence supports that fish oil provides preventative or ameliorative effects against thrombotic disease, the mechanisms responsible for this association are not understood and are further complicated by contrasting reports. Fish oils are a rich source long chain omega-3 polyunsaturated fatty acids including eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), however it is not clear whether the anti-thrombotic effects are due to EPA or DHA or whether both are equally effective. In the available literature relating fish oil and platelet aggregation, wide variability in terms of dosage, concentration ratios, study design, subject characteristics and gender inequality are apparent, hence there is discrepancy regarding the effect of fish oils on platelet activity. Consequently, the anti-thrombotic potential of fish oil supplementation is controversial and largely disregarded by the medical community.

This dissertation investigated the independent effects of EPA and DHA on platelet and coagulant activity. A series of three controlled studies were undertaken to elucidate the mechanisms by which EPA and DHA influence hemostatic parameters with the hope to resolve the existing controversy. The ultimate and unifying theme
of these studies was to provide a safe and efficacious approach to optimise cardio-
protection via anti-thrombotic potential of EPA versus DHA.

Firstly, an *in vitro* investigation was carried out that compared the effects of EPA
with DHA on platelet aggregation in healthy male and female subjects. The
inhibition of platelet aggregation by EPA/DPA/DHA was equally effective and
correlated with lag time; however most strikingly the results were influenced in a
gender-specific manner. These observations suggest that interactions between sex
hormones and fish oils exist to influence platelet response differentially.

With a new perspective of gender bias effects, an acute supplementation study
monitored the platelet responses up to 24 hours after consumption of a single dose of
an EPA versus DHA-rich oil capsule in thirty male and female subjects. The kinetics
of the EPA and DHA supplement on platelet activity was examined according to
gender stratified treatment. Subgroup gender analysis showed that the anti-
aggregatory effects of EPA were predominately evident in males while female
platelets were more responsive to DHA. The marked decrease in platelet aggregation
with EPA supplementation was paralleled with a reduction in platelet microparticle
activity in the male subjects only, and an inverse relationship between testosterone
levels and platelet responses were observed. Findings from this study reflected the *in
vitro* observations and suggest that EPA and DHA inhibit platelet aggregation via
independent pathways compounded by sex hormonal influences.

Confirmation of gender-specific platelet responses with omega-3 fatty acid
supplementation was achieved in a chronic supplementation study involving ninety-
four healthy male and female subjects. Subsequently, this four week dietary
intervention trial demonstrated that the anti-thrombotic potential is apparent with
longer term exposure to EPA/DHA and explored the mechanistic pathways.
Significant interactions between gender and treatment were observed; the effects of
EPA were specific in reducing platelet aggregation and specific coagulation factors
in males, whereas no effects were observed in the female cohort. Conversely, the
effects of DHA were unique to females with a similar decrease in platelet
aggregability. Interactions between sex hormones with coagulation factors and
retention of EPA and DHA in plasma were also observed.
In conclusion, the study findings presented in this thesis provide evidence that the effects of EPA and DHA on platelet aggregation are apparent; the effects are neither shared nor complementary, rather they are gender-specific. Furthermore, the results herein may explain the existing controversy between fish oils, platelets and thrombosis that have intrigued clinical investigators for several decades. With respect to thrombotic disease risk, males would likely benefit more from supplementation with EPA while females are more responsive to DHA. The significance of these findings allows optimal cardio-protection tailored for both gender groups offering a safe and efficacious non-pharmacological approach.
Thesis structure and chapter overview

This thesis consists of five peer-reviewed publications that have been published in quality scientific journals and one publication in the form of a book. The thesis begins with an introduction and review of the literature (Chapter 1) followed by the methodology undertaken in the conduct of the research (Chapter 2). The background, study design and methods, results, discussion and implications of the research conducted for this thesis are then presented as a series of five research papers (Chapter 3 to 5). This thesis and the papers present work form a body of research comprised of five key components: a literature review (i) followed by the methods (ii), leading to the three subsequent human research studies; an *in vitro* investigation (iii), an acute supplementation study (section iv) and a long term dietary intervention study (v). A brief overview of each component is provided below. An overall discussion of the findings from the body of research and its implications are provided as the final chapter of the thesis (vi).

(i) Literature review: Chapter 1

Chapter one begins with an introduction of the hemostatic system followed by early basic research on LCn-3PUFA to the contemporary research of the current and emerging health issues. Excerpts from this chapter have been published:

*Publication 1:*


*Publication 2:*

This chapter also explores the cardiovascular sex differences and controversy in the literature surrounding platelet aggregation and LCn-3PUFA, and ultimately introduces the premise of this dissertation. An up-to-date review discussing the sex relevant differences in this context is provided accompanied by the available literature; essentially highlighting the need for future sex-specific analyses to be conducted. The chapter concludes with the hypothesis and ultimate aims to be tested in this thesis.

(ii) Methods: Chapter 2
The study design and methods employed to undertake all data, scientific laboratory and statistical analyses are described in this chapter.

(iii) In vitro investigation: Chapter 3
Chapter three describes an in vitro investigation designed to assess the effectiveness of EPA, DPA and DHA to inhibit platelet aggregation in healthy human subjects. The investigation compared platelet aggregation in human whole blood samples incubated with various concentrations of the individual LCn-3PUFA; EPA, DPA and DHA. As discussed in my original hypothesis (section 1.2.1), this study was initially designed to examine the individual LCn-3PUFAs on platelet aggregation.

The content of this chapter is covered by:

Publication 3:

(iv) Acute supplementation study: Chapter 4
This chapter describes Study 2; a randomised, blinded placebo-controlled trial where platelet function of healthy subjects were measured at various time intervals over a 24 hour period following dietary supplementation of the fish oil concentrates of low
versus high EPA to DHA ratios or placebo. Since DPA possessed no unique effects on platelet aggregation in study 1, further studies were focused on EPA and DHA only.

The content of this chapter is covered by:

**Publication 4:**

**Publication 5:**

(v) **Long-term dietary intervention study: Chapter 5**
Chapter five describes Study 3; a double-blinded, randomised, placebo-controlled trial over a 4 week dietary intervention period. Platelet function, full blood count parameters, procoagulant activity, biomarkers of platelet activity, coagulation and fatty acid profiles of healthy subjects were measured at baseline and post-intervention following 4 week supplementation of fish oil concentrates of low versus high EPA to DHA ratios or placebo.

The content of this chapter is covered by:

**Manuscript 6:**
**Manuscript 7:**

Chapter One: Introduction and literature review

Excerpts from this chapter have been published:

Phang, M., Lazarus, S., Wood, L.G & Garg, M.L; ‘Diet and thrombosis risk: Nutrients for prevention of the disease’; Seminars in Thrombosis & Hemostasis, April 2011; 37; 3; 199-208

1.1. Background and context

1.1.1. Cardiovascular and thrombotic disease

The term cardiovascular disease (CVD) is generally broad; commonly it centres on atherosclerosis, focusing on cholesterol levels, plaque formation and the narrowing and hardening of the arteries [1]. Extensions to other cardiovascular complications and diseases are either a cause or consequence of atherosclerosis; these including hypercholesterolemia [1], diabetes [2], hypertension, thrombosis and stroke [3]. Coronary heart disease (CHD) or coronary artery disease (CAD) involve narrowing of the arteries resulting in depleted oxygen supply and ultimately damage to the myocardium. The complete blockage of blood supply to the heart by a thrombus extends to a myocardial infarction (MI) [4]. A thrombus is a solid mass of platelets, red blood cells and fibrin varying in composition depending on their site of formation and shear stress conditions. The thrombus undergoes constant change and its eventual fate is decided by a balance of forces leading to its deposition and removal, including embolisation and fibrinolysis [5].

Stroke involves either formation of a thrombus in a cerebral artery resulting in a cerebral infarction or the rupturing of a cerebral artery [6]. Thus CVD in this broad sense is not classified as a single disease, but rather an extensive web of interconnected diseases hence further broadening the scope of the relevant pathophysiology. The development of atherosclerosis and thrombosis are interrelated and notably play a pivotal role in CHD and stroke. Collectively, cardiovascular diseases place an overwhelming burden upon the community and institutions in terms of health, social, economic and emotional costs and sadly they are the leading cause of morbidity and mortality in westernized societies [1]. On a global scale, 16.7 million (29%) of the 57 million deaths annually are related to cardiovascular conditions [3]; atherosclerosis being the underlying cause of approximately 50% of all deaths in westernised societies [1]. It should be noted that atherosclerosis without thrombosis is in general considered a benign disease, survival rates are indeed dependent on prevention of thrombus-mediated complications [7]. The complications of thrombosis are represented by the formation or presence of a thrombus within the
artery or vein obstructing the circulation of blood [8]. The ramifications of arterial thrombosis can result in cerebral or myocardial infarction causing immediate morbidity or mortality [9]. Approximately 75% of thrombi responsible for acute coronary syndromes (ACS) are precipitated by plaque rupture whereby thrombogenic material is exposed to the circulation [7]. Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), affects approximately 1-2 per 1000 of the general population [10]. In addition, an approximate 6% of DVT cases and 12% of PE cases results in mortality within the first month of diagnosis alone [11]. With respect to morbidity, 23-60% of DVT patients also risk development of postphlebitic syndrome [12]; a potentially debilitating long-term sequelae of recurrent DVT characterized by chronic persistent pain and inflammation in the affected limb [13]. Platelet aggregation secondary to atherosclerotic plaque disruption results in thrombus formation thus increased platelet aggregation may induce formation of platelet-rich thrombi occluding blood vessels leading to cardiovascular complications and mortality [14]. Hence platelets represent a key element in the pathogenesis and development of CVD and indeed increased platelet aggregation is the major cause of cerebral and myocardial infarction, hypertension and arterial and deep vein thrombosis [15-18].

1.1.2. Platelet discovery

Of the pioneer investigators, Max Schultze, William Osler, Georges Hayem and Julius Bizzozero received recognition for being the first to identify and describe the blood platelet. In 1865, Schultze published the first accurate and convincing description of platelets and recognised them as a normal constituent of the blood, not an artefact [19]. Osler, in 1873 further identified platelets as disk-like structures that circulated throughout the blood stream, aggregating upon removal. Possibly the most original observation documented by Olser was that platelets did not exist as aggregates in the blood, but in the form of isolated corpuscles [20]. Hayem, in 1877 helped establish that platelets were a distinct cellular entity, provided a firm histological basis for platelets and noted their role in coagulation [21]. In 1881, Bizzozero further characterized platelets anatomically and in well-planned experiments, he showed that they were the first component of the blood to adhere to damaged blood vessel walls in vivo [19]. Though working independently over an 8-
year interval their works collectively provided observations that established the foundation for platelet biology.

1.1.3. Platelet biology
Platelets are small specialised, discoid, anuclear cell fragments derived from megakaryocytes in the bone marrow and degraded in the spleen and liver [22]. They are the smallest of cells in circulation measuring 2-5µM in diameter and 0.5µM in thickness [23, 24]. The normal platelet concentration is 150,000–350,000/µL and platelets have an average life span of 8-12 days [25]. The platelet membrane contains tiny folds providing additional surface area for platelet spreading [26]. Small openings on the surface create channels of the open canalicular system (OCS) that in conjunction with the actin-like submembrane filaments play a crucial role in shape change and translocation of receptors to the outer surface of the cell [27]. A coil of microtubules within the cytoplasm wraps around the circumference of the cell providing cytoskeletal support along with an actomyosin filament system involved in shape change, internal transformation and contraction as well as the retraction of hemostatic plugs [28].

1.1.4. Platelet lipid membrane
The lipid membrane of platelets is comprised of approximately 75 % phospholipids (PL), 20% neutral lipids and 5 % glycolipids [29]. The PLs include sphingomyelin, plasmalogens, lysophospholipids and glycerophospholipids. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant glycerophospholipids, each constituting approximately 35 % of the PL content. Phosphatidylserine (PS) constitutes approximately 13 %, and phosphatidylinositol (PI) accounting for less than 5% [29, 30]. Phosphatidylcholine (PC) and sphingomyelin are found predominately in the outer monolayer, while the anionic phospholipids PS and PE are located in the inner monolayer of the platelet membrane [31]. The fatty acid (FA) content varies widely in the different PL classes predisposed by the FA content in plasma which in turn is reflected by the dietary FA intake [32]. The lipid types and their distribution within the membranes may be unique to different cell types but are clearly altered by the availability of dietary lipids, particularly of the polyunsaturated fatty acid (PUFA) class. Lipid composition greatly influences membrane fluidity, the formation of platelet receptors and
subsequent binding of ligands to their receptors as well as activation of intracellular signalling pathways [33]. Thus modulation of dietary FA intake is of significance given that such changes in FA composition are considered the main features responsible for alterations in platelet function driven by lipid alterations exerting control over cellular function.

1.1.5. Platelet surface
In resting platelets, the asymmetric PL organisation and composition is maintained by an aminophospholipid translocase promoting inward transport specific for PS and PE [34]. The loss of this PL transmembrane asymmetry, and the consequent exposure of PS and PE on the platelet surface, is likely to be mediated by a Ca$^{2+}$-dependent phospholipid scramblase activity and concomitant inhibition of aminophospholipid translocase activity [34, 35]. The Glycocalyx, (thick exterior coat of the platelet plasma membrane) containing glycoproteins (GP), glycolipids and adsorbed plasma proteins provides a transfer point for plasma proteins as they are taken up into secretory granules by endocytosis, and producing a net negative surface charge mainly due to sialic acid residues attached to proteins and lipids [36, 37]. This electrostatic repulsion is likely to prevent resting platelets from attaching to each other and to the negatively charged endothelium [37].

1.1.6. Platelet receptors
An array of transmembrane receptors covers the platelet membrane, including many integrins ($\alpha_{IIb}\beta_3, \alpha_5\beta_1, \alpha_\beta_1$, $\alpha_\gamma\beta_3$), leucine-rich repeated (LRR) receptors (Glycoprotein [GP] Ib/IX/V, Toll-like receptors), G-protein coupled transmembrane receptors (GPCR) PAR-1 and PAR-4 thrombin receptors, P2Y$_1$ and P2Y$_{12}$, ADP receptors, TP$\alpha$ and TP$\beta$ TXA$_2$ receptors), proteins belonging to the immunoglobulin superfamily (GP VI, Fe$\gamma$RIIA), C-type lectin receptors (P-selectin) and a miscellaneous of other types (CD63, CD36, P-selectin ligand 1, TNF receptor type, etc). Some receptors are expressed exclusively on platelets while others are also shared by other cell types [38]. It is well established that the major platelet receptors play a prominent role in hemostasis, allowing specific interactions and functional responses of vascular adhesive proteins and of soluble platelet agonists (Table 1-1). In particular, the GP VI immunoglobulin superfamily member and the integrin $\alpha_\gamma\beta_3$
(GPIa/IIa) are the two platelet receptors that have been demonstrated to bind directly to collagen while the GP Ib/IX/V complex is the major platelet receptor mediating interaction with von Willebrand Factor (vWF). The expression level of GP VI and $\alpha_2\beta_1$ are controlled by common polymorphisms and correlates with the in vitro rapidity in platelet adhesion and responsiveness to collagen [39, 40]. The GP Ib/IX/V complex (GPIbα, GPIbβ, GPIX and GPV) having bound to von Willebrand Factor (adhesive glycoprotein) causes platelet activation through activation of protein kinase C, phosphatidylinositol 3-kinase and tyrosine kinases, resulting in an increase in intracellular calcium and to inside-out signalling through GPIIb/IIIa followed by aggregation [41]. In addition to vWF, the GP Ib/IX/V complex also binds to other adhesive proteins (collagen, thrombospondin-1), thrombin and coagulation factors (FXI, FXII) while further playing a substantial role in platelet interaction with activated endothelial cells and leukocytes via binding of P-selectin and $\alphaM\beta_2$, respectively [38]. GPIIb/IIIa ($\alpha_{IIb}\beta_3$) is the major surface receptor, present in 40 000 to 80 000 copies on the plasma membrane of a resting platelet while another 20 000 to 40 000 copies are localized inside the platelets and are capable of joining the platelet surface membrane upon platelet secretory activation [42-44].

Table 1-1. Platelet plasma membrane receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIa</td>
<td>Fibrinogen, vWF</td>
<td>Platelet aggregation</td>
</tr>
<tr>
<td>GPIb/IX/V</td>
<td>vWF, thrombin</td>
<td>Platelet adhesion</td>
</tr>
<tr>
<td>PAR-1, PAR-2</td>
<td>Thrombin</td>
<td>Platelet activation, platelet aggregation</td>
</tr>
<tr>
<td>P2Y$<em>1$, P2Y$</em>{12}$</td>
<td>ADP</td>
<td>Platelet activation, platelet aggregation</td>
</tr>
<tr>
<td>GPVI</td>
<td>Collagen</td>
<td>Platelet activation, platelet aggregation</td>
</tr>
<tr>
<td>P-selectin</td>
<td>P-selectin glycoprotein ligand (PSGL)</td>
<td>Cell adhesion, WBC tethering and binding</td>
</tr>
</tbody>
</table>

Abbreviations: ADP, adenosine diphosphate; GP, glycoprotein; PAR, protease activated receptor; vWF, von Willebrand Factor; WBC, white blood cell
1.1.7. Platelet granules

Platelets contain three unique types of secretory organelles, α-granules, dense core granules and lysosomes. The α-granules are the most abundant with an approximate 40 to 80 per platelet and contain hemostatic proteins, fibrinolytic regulators and adhesion molecules such as fibrinogen, vWF, and growth factors [36] (Table 1-2). The dense granules are smaller, less abundant with approximately 1 to 1.4 per platelet, and contain proaggregatory factors including adenosine 5'-diphosphate (ADP), calcium, and 5-hydroxytryptamine (serotonin) [36]. During activation, the granules are centralized and their contents are discharged into the lumen of the open canalicular system, from which they are then released to the exterior, this process termed the ‘release reaction’[45]. Platelet degranulation occurs following platelet activation by specific ligands including thrombin, collagen, and thromboxane A2 (TXA2) which act through G protein-coupled receptors. The granule secretion is graded and dependant on the nature and concentration of the original stimulus/stimuli whether strong (eg. collagen) or weak (eg. ADP) [46]. Platelet degranulation typically occurs through a Gq protein-coupled mechanism, plasma membrane phosphatidylinositol 4,5 bisphosphate (PIP2) is cleaved into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3), which activate protein kinase C (PKC) and increase intracellular Ca2+. Direct increases in cytoplasmic Ca2+ along with the synergistic effects of PKC result in platelet degranulation and the initiation of a hemostatic response [27]. The release of these factors results in a precisely regulated series of events influencing a variety of biological functions including cell adhesion, cell aggregation, chemotaxis, cell survival, proliferation, coagulation, proteolysis, and cell recruitment [47].

Table 1-2. Platelet α-granule contents in hemostasis

<table>
<thead>
<tr>
<th>Adhesion molecules</th>
<th>P-selectin (CD62P), von Willebrand Factor (vWF), thrombospondin, fibrinogen, fibronectin, integrin α1bβ3, integrin αvβ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation pathway</td>
<td>Factor V, multimerin, Factor VIII</td>
</tr>
<tr>
<td>Fibrinolytic pathway</td>
<td>Plasminogen, plasminogen activator inhibitor-1 (PAI-1)</td>
</tr>
</tbody>
</table>
1.1.8. Primary hemostasis

Since the initial investigations that established the foundation of platelet biology in the early 1870’s, almost 140 years onwards we are now able to confidently identify platelets and establish their function in hemostasis and role in CVD. Although, as early as 1974, platelet emboli were detected in the coronary microcirculation in the early stages of ACS patients. Later in 1984, it was shown by autopsy procedures that in the majority of sudden death cases due to MI, thrombi were present. The primary function of platelets is to arrest haemorrhage at sites of vascular injury with the ability to rapidly form platelet-rich thrombi [14]. Thus platelets play a central role in hemostasis and thrombosis by orchestrating the crucial set of events associated with the maintenance of blood vessel integrity. Hemostasis is the complex physiological defence against blood loss involving regulation of clot formation [48]. This process is comprised of platelet aggregation and coagulation, also termed primary and secondary hemostasis respectively [49]. Primary hemostasis results in the formation of the platelet plug via platelet aggregation [50] which is initiated through the sequential events of platelet adhesion, activation and secretion of aggregatory agents [51]. Secondary hemostasis proceeds simultaneously and is responsible for activation of the coagulation cascade by providing interactions and a procoagulant surface between platelet membranes, enzymes and clotting factors [49] (Figure 1-1).
Figure 1-1. **Platelets in primary and secondary hemostasis;**

**A:** Exposed tissue factor and phospholipid activates the coagulation cascade culminating in the formation of thrombin. Activation of thrombin converts fibrinogen to fibrin forming a mesh network work and stabilising the hemostatic plug. **B:** Platelets adhere to the exposed collagen and vWF in the subendothelium resulting in platelet activation and shape change. Activated platelets release stored granule contents (eg. ADP, TX) which recruits other platelets and triggers platelet aggregation forming a hemostatic plug. From: Kumar and Robbins (2007) [52].
1.1.8.1. **Platelet adhesion**

The hemostatic process is activated upon vascular breach, exposing collagen and vWF in the subendothelium to circulating platelets [53]. Platelets adhere to sites of vascular injury through interactions with the exposed subendothelial matrix and the adhesion proteins. Platelets adhere to vWF via interactions with glycoprotein (GP) Ib/IX/V complex on the platelet membrane allowing contact with collagen fibrils through the GPIa adhesion receptor [54]. Collagen-mediated platelet activation stimulates the expression of integrin receptors $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) and $\alpha_2\beta_1$ (GP Ia/IIa) required for irreversible platelet adhesion. Activation of $\alpha_{IIb}\beta_3$ causes a conformational change that enables it to bind fibrinogen [55, 56]. Platelet activation causes mobilization of these intracellular fibrinogen-GP IIb/IIIa complexes to the platelet surface in a secretion-dependent manner [57, 58].

1.1.8.2. **Platelet activation**

Contact with collagen on the platelet surface receptor site results in activation of the adherent platelets causing morphological change from smooth disks to irregular sphere and pseudopodia extensions [59, 60]. The principal agonists of platelet activation are collagen, thrombin and thromboxane $A_2$ (TX$A_2$) [61]. These mediators will bind to their respective receptors on the platelet surface stimulating the release of factors from intracellular storage granules [62]. Activated platelets also release arachidonic acid (AA) stored in the platelet membrane phospholipids, which is then converted to TX$A_2$ via the cyclo-oxygenase (COX) pathway [63]. Two isoforms of the COX enzyme exists; COX-1 is constitutively expressed in most cells, whereas COX-2 is induced by stimulation with various growth factors and cytokines.

Soluble P-selectin (P-sel) is shed from activated platelets and endothelial cells to support recruitment of leukocytes and platelet adhesion [64]. Secretion of TX$A_2$ along with other granule contents (eg. ADP, calcium, thrombin) activates other nearby platelets and prepares platelet surfaces for reactions with the coagulation proteins in secondary hemostasis [65]. Dependent on the activator, the normal asymmetric distribution of the platelet phospholipid membrane is disrupted, resulting in exposure of an anionic procoagulant surface (PS) at which the coagulation factors are activated to generate thrombin which then catalyses fibrinogen formation [31, 66, 67]. In addition to providing the procoagulant phospholipid surface and binding sites
for several coagulation factors, platelets contribute to coagulation activity by releasing several compounds, such as FV, FXIII, fibrinogen and vWF [66].

1.1.8.3. Platelet aggregation

The collagen-activated platelet membranes expose GP IIb/IIIa receptors which allow circulating fibrinogen to bind to their surfaces. GPIIb and GPIIIa require Ca$^{2+}$ to maintain their heterodimeric structure [68] and the receptor complex undergoes a Ca$^{2+}$-dependent conformational change after platelet activation, strongly facilitating binding of fibrinogen and VWF [47]. The outcome is cross-linking of GPIIb/IIIa receptors on adjacent platelets and subsequent platelet aggregation where activated platelets become attached to one another and stick to those adhering to collagen and vWF [50]. A bridging network of platelet aggregates is created forming the primary haemostatic plug. Under these conditions, secondary hemostasis is activated where thrombin is formed in the coagulation pathway to convert soluble plasma fibrinogen into an insoluble fibrin clot [69]. The production of thrombin in turn stimulates platelet aggregation [70] resulting in further growth and development of the thrombus.

1.1.9. Secondary hemostasis

In resting platelets, the outer leaflet of the plasma membrane consists mainly of neutral phospholipids, whereas the negatively charged phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are almost exclusively present in the inner leaflet [71]. The localization of these anionic phospholipids on the inner side of resting platelets is a crucial mechanism to prevent inappropriate coagulation [72]. The loss of this phospholipid transmembrane asymmetry via platelet activation, allows the exposure of PS and PE, consequently providing a procoagulant surface to accelerate several steps in the coagulation cascade.

1.1.9.1. Coagulation

The coagulation pathway is composed of circulating zymogens coupled with a series of serine protease enzymes and their co-factors that interact on an anionic phospholipid surface. This process consists of a recurrent theme involving the rapid assembly of active coagulation factors to produce an efficient molecular cascade reaction ultimately resulting in cross-linking fibrin [65]. The cascade model of
Coagulation was originally proposed in 1964 [73, 74] and was based on the concept of distinct intrinsic and extrinsic coagulation pathways (Figure 1-2) reflected by the clinical laboratory tests; activated partial thromboplastin time (APTT) and prothrombin time (PT), respectively. Albeit this concept remains useful in vitro for diagnostic purposes, in vivo these pathways are integrated, owing to the appreciation now that tissue factor–factor VIIa complex (TF–VIIa) activates both factor IX and factor X (Figure 1-3) [75]. Thus the significance of separate intrinsic and extrinsic pathways has been questioned, with rather current emphasis shifted to the role of cellular components in regulation the coagulation pathway [76-78].

**Figure 1-2. Traditional model of the coagulation cascade.**
In the traditional model, the intrinsic and extrinsic pathways are reflected by the clinical laboratory tests; activated partial thromboplastin time (APTT) and prothrombin time (PT) respectively before the final common pathway. F, factor; TF, tissue factor; PL, phospholipids Adapted from: Hoffman and Monroe (2001) [77].
Figure 1-3. Revised model of the coagulation cascade.
TF-FVIIa complex activates both FIX and FX. F, factor; TF, tissue factor
Adapted from: Hoffman and Monroe (2001) [77].

1.1.9.2. Platelet procoagulant activity
In recent years, it is increasingly appreciated that formation of procoagulant membrane surfaces involves exposure of anionic phospholipids at the platelet’s outer surface and that this process is tightly associated with shedding of microparticles from the platelet plasma membrane [66, 79]. Platelet membranes contain two anionic phospholipids, phosphatidylserine (PS) and phosphatidylinositol (PI). Theoretically, any anionic phospholipid is capable of providing a catalytic surface; however membranes containing PS exhibit the highest procoagulant activity. In contrast to other anionic phospholipids, PS is the only lipid that retains its catalytic properties, irrespective of the ionic strength of the medium or the net charge of the lipid surface [67, 71, 80].

1.1.9.3. Membrane phospholipids
The loss of transmembrane asymmetry following platelet activation results in exposure of an anionic PL surface. Phosphatidylserine exposure on the outer membrane of the platelet provides the surface at which clotting proteins are activated to generate thrombin [67, 71]. Two consecutive reactions of the coagulation pathway are dramatically accelerated in the presence of an anionic PL surface. Essentially, the PS surface provides high affinity attachment sites for cofactors VIIIa and Va and their respective enzymatic factors IXa and Xa. [81]. The first is the
conversion of the zymogen factor X into an active protease Xa catalysed by the tenase complex, consisting of factor IXa in conjunction with factor VIIIa. Subsequently factor Xa assembles with its cofactor Va on the platelet surface forming the prothrombinase complex. Prothrombin bound to GPIIb/IIIa is efficiently cleaved by factor Xa resulting in the release of thrombin [82]. The extrinsic tenase complex activates FX at a 50-fold higher rate than the TF-VIIa complex, whereas it is estimated that the presence of PL stimulates the prothrombinase complex to convert prothrombin to thrombin at a rate of $10^3$ to $10^5$-fold faster than a physiological concentration of FXa alone [83, 84]. Thrombin is capable of further activating platelets through specific receptors (PAR-1 and -4) [85] causing activation of GPIIb/IIIa receptors. As well as thrombin up-regulating its own generation through activation of FV, FVIII, and FXI [86]. Finally thrombin stabilizes the platelet plug by conversion of fibrinogen from a soluble plasma protein into an insoluble fibrin clot [87].

1.1.9.4. Platelet-derived microparticles

During the process of PS exposure, activated platelets change shape and form membrane blebs which are then fragmented off from the remnant platelet as microparticles (MP) [88, 89]. With respect to activated platelets, MPs are shed from the outer membrane thus providing additional procoagulant phospholipid surface to enhance the formation of the intrinsic tenase and prothrombinase complexes [90, 91]. Thus platelets can foster the generation of thrombin not only on their activated surface but also on shed MPs (Figure 1-4). In addition, MPs are highly mobile and capable of mediating these effects at areas distant from the initial site of platelet activation [92]. MPs are also capable of transferring AA further promoting further activation and up-regulation of adhesion molecules [93].
Figure 1-4. Generation of microparticles and their procoagulant activity. Activated platelets shed MP from the outer membrane thus providing additional procoagulant phospholipid surface to enhance the formation of the intrinsic tenase and prothrombinase complexes. Adapted from: Nomura et al (2001) [94].

1.1.10. Fibrinolysis
The final stage of hemostasis is the activation of the fibrinolytic system to dissolve the fibrin clot. Activation of coagulation ultimately generates thrombin, which results in thrombus formation by conversion of fibrinogen to fibrin and by platelet activation. Both coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, inhibitors, cofactors and receptors. The key reaction in fibrinolysis is the activation of the proenzyme plasminogen (PLG) to the serine protease plasmin. Plasminogen binds via its lysine-binding sites to specific lysines in the fibrin molecules [95]. Fibrinolytic protease plasmin is converted from PLG by tissue plasminogen activator (tPA) as well as urokinase plasminogen activator (uPA). Plasmin cleaves both tPA and uPA through a positive feedback mechanism transforming them from single chain to more active two-chain polypeptides [15].

Inhibition of fibrinolysis occurs at the level of the activators by plasminogen activator inhibitors (PAI) or at the level of plasmin (mainly by α2-antiplasmin). Physiologic fibrinolysis is highly fibrin-specific as a result of specific molecular interactions between PA, plasminogen, fibrin, plasmin, and α2-antiplasmin. Fibrin is
the major plasmin substrate and regulates its own degradation by binding PLG and tPA on its surface, thereby localising and enhancing plasmin generation. Once formed, plasmin cleaves fibrin, generating soluble degradation products [15] (Figure 1-5). At least four PAI have been identified including PAI-1, PAI-2, PAI-3 and protease nexin [96]. Although both PAI-1 and PAI-2 play an eminent role in the regulation of fibrinolysis, PAI-2 inhibits only uPA while PAI-1 inhibits both tPA and uPA. Protease nexin inhibits uPA, thrombin and plasmin while the biological role of PAI-3 in modulating fibrinolysis still remains unclear [97]. The PAI-1 molecule is the major PAI in the circulation, representing 60% of plasminogen activation inhibition in plasma. Thus PAI-1 is a fast-acting inhibitor of fibrinolysis, which alters the thrombotic balance in favour of occlusion. It has been reported that levels of PAI-1 are elevated in young men surviving a myocardial infarction and also predict recurrent events [98, 99] hence considered a common denominator in CVD.

**Figure 1-5. The fibrinolytic system.** Plasminogen is converted to plasmin by tPA and u-PA, which is inhibited by PAI and α2-antiplasmin. Once formed, plasmin cleaves fibrin, generating soluble degradation products. From: Kumar and Robbins (2007) [52].
1.1.11. Principal physiological agonists of platelet activation and Aggregation

1.1.11.1. Collagen
Collagen is important for platelet adhesion and subsequent activation, platelet collagen interactions are initiated indirectly by interaction of GPIb/IX/V with collagen-bound vWF [100]. Platelets firmly adhere following generation of platelet activating signals initiated by GP VI [101]. GP VI mediates collagen signalling [101] and is responsible for mobilizing intracellular calcium stores required for the formation of secure platelet interactions [102]. Although GP VI receptors are constitutively expressed on the platelet surface, additional GP VI molecules can be recruited following platelet activation [99, 101]. Coincidently, it has been reported that platelet surface density of GP VI is related to the incidence of acute coronary events in patients with acute coronary syndrome compared to those with stable coronary artery disease [103]. The GP Ia/IIb collagen receptor further amplifies the response driven by GP VI [104]. Thus collagen is an important agonist in platelet aggregation studies performed in vitro as a means of recreating the in vivo environment where the, damaged vascular endothelial surface exposes subendothelial collagen fibres initiating the aggregation response [105].

1.1.11.2. Adenosine diphosphate
Adenosine diphosphate (ADP) is a relatively weak mediator of platelet aggregation as it stimulates minimal granule secretion and must be continuously bound to its platelet receptor for fibrinogen binding to occur. ADP forms large aggregates under low shear stress that dissociate into microaggregates under more normal shear resulting in a transient aggregatory response stress [106, 107]. Although considered a weak platelet agonist, it plays a critical role in amplifying the effects of other platelet agonists. ADP interacts with the G protein-coupled P2Y family, receptors P2Y1 and P2Y12. Receptor P2Y1 contributes to initial platelet activation while the P2Y12 receptor acts as a co-stimulus in the presence of low concentrations of other agonists [38].
1.11.3. **Thrombin**

Thrombin is considered to be the most potent biological activator of platelets; promoting fibrin generation and thrombus formation under all shear stress conditions [61] and is able to rapidly increase cytosolic calcium inducing shape change, degranulation and aggregation [108]. Thrombin-mediated activation of platelets occurs mainly through interactions with protease-activated receptor (PAR) family members, with human platelets expressing PAR-1 and PAR-4 [109].

1.11.4. **Thromboxane A\(_2\) (TXA\(_2\))**

Activated platelets release arachidonic acid (AA) stored in the platelet membrane phospholipids, which is then converted to TXA\(_2\) via the COX pathway through the oxidation of AA [63]. Upon synthesis, it diffuses across the platelet membrane and activates other recruited platelets, thus favouring the growth of the platelet plug. TXA\(_2\) binds to G-coupled TP receptors [110] that stimulate phospholipase C to increase cytosolic Ca\(^{2+}\) levels [111, 112]. Though TXA\(_2\) has been identified as both a potent and weak agonist, regardless of its strength, it is considered an important positive feedback regulator in the activation and recruitment of platelets to the growing thrombus [112]. The production of TXA\(_2\) has also been identified as an important mediator of granule release required for the ‘secondary wave’ of aggregation to occur [113].

1.11.5. **Platelet procoagulant activity / Platelet-derived MP**

The activated platelet membrane exposing anionic membrane phospholipids permits the binding of clotting proteins Factor V and X complex, leading to greatly increased catalytic activity of these plasma factors and ultimately thrombin generation [81]. Microparticles are continuously shed from the platelet membrane surface, however their generation is significantly enhanced upon platelet activation [114] by physiological agonists [115] and high shear stress [116]. Similar to activated platelets, MPs exert procoagulant effects due to the presence of surface anionic membrane phospholipids that enhance the formation of the intrinsic tenase and prothrombinase complexes [90, 91]. Interestingly, MPs have been reported to have 50 to 100 times higher specific procoagulant activity than activated platelets [92].
**1.1.11.6. Soluble P-selectin (CD62P)**

The glycoprotein P-selectin (P-sel) is a cell adhesion molecule expressed on activated platelets present in a thrombus which supports the recruitment of leukocytes and platelet adhesion [117]. P-selectin expression on the surface of activated platelets mediates platelet-endothelial interactions. Binding of platelet P-sel to the P-sel glycoprotein ligand-1 (PSGL-1) on monocytes initiates formation of platelet-monocyte aggregates and enhances the production of growth factors, proteases, and tissue factor [118, 119]. It has become apparent that platelet adhesion to the vessel wall is not always initiated by endothelial injury or extracellular matrix protein exposure [118]. Platelet activation is associated with increased wall thickness of the carotid artery [120] and indeed platelets have been demonstrated to adhere to uninjured but activated endothelium [121, 122]. Furthermore, P-selectin expression has been reported to be significantly elevated in patients with ACS compared to subjects with stable angina pectoris [103]. Thus, current data reveal that platelet P-selectin is a key mediator of inflammation contributing to atherosclerotic plaque formation [108].

**1.1.12. Hemostasis versus thrombosis: Maintaining the balance**

Platelets are regarded as the preeminent cells involved in physiological hemostasis and pathological thrombosis. In simple terms, thrombosis can be considered as an aberration of normal hemostasis. The primary function of hemostasis is to prevent loss of blood from damaged blood vessels [123]. Thrombosis, the formation of a thrombus in a blood vessel obstructing blood flow in the circulation can potentially contribute to fatal events. In general, thrombus formation is a normal body mechanism to repair injury to blood vessel endothelial surfaces involving the aggregation of platelets, ultimately resulting in the formation of a clot composed of fibrin strands. However, the formation of a thrombus in a blood vessel may result in the obstruction of blood flow in the circulation with potentially fatal consequences.

Platelets are unable to distinguish between damaged endothelium and pathological lesions, such as those present in atherosclerotic vessels, which can result in thrombosis. Both hemostasis and thrombosis have common modifiers and are influenced by the strength of the aggregatory stimulus. Normal platelet response can
be altered by an imbalance of increased potent pro-aggregatory agents such as thromboxane $A_2$ (TXA$_2$) or diminished anti-aggregatory substances [124].

As aforementioned, normal hemostasis a process that is comprised of a scheme of events involving the three main phases:

(i) Platelet adhesion and aggregation: formation of an unstable platelet plug
(ii) Coagulation: consolidation of the platelet plug with fibrin
(iii) Fibrinolysis: degradation of the stable fibrin clot

Thrombosis is a distortion of the hemostatic process when the regulatory mechanisms are overwhelmed, resulting in excess clotting [125], while impaired hemostasis may lead to abnormal bleeding and risk of haemorrhage [126]. Thus it is imperative to maintain the balance between hemostasis and thrombosis.

1.1.13. Thrombosis and other CVD risk factors

It is without doubt that great strides have been made in the understanding of the mechanisms of atherogenesis that have established the irrefutable association of clear causality between the classic risk factors and CVD. The major modifiable risk factors for CVD have been identified from large prospective studies such as the Framingham Heart Study [127] and the Seven Countries Study [128] which include hypertension, hyperlipidemia, smoking, obesity, diet and diabetes mellitus. However, CV events can also occur even in subjects without these established risk factors and on an individual basis, the prediction of the risk of CVD from levels of blood pressure, lipids, and smoking are poor [129]. More interestingly there is evidence that occlusive thrombi are to be found in almost all cases of acute myocardial infarction and in the majority of sudden cardiac deaths [130, 131].

Over the last two decades, it is increasingly apparent that inflammation and thrombosis are the intermediate effectors between atherogenic risk factors and CVD [132, 133]. Although substantial progress has been made in understanding the pathophysiology of thrombosis, it still remains the final pathway to disease and death in some of our most common pathologies. However, it is evident that the events involved in primary hemostasis leading to the development of thrombosis can be suppressed to reduce the risk of disease. Indeed, the mechanisms that protect against
thrombosis include decreased cell adhesion molecule expression, decreased aggregatory stimuli ($\text{TXA}_2$), increased circulating inhibitors, dilution and clearance of activated coagulation factors, and inhibition of platelet aggregation [53]. Consequently, several inflammatory biomarkers, hemostatic and thrombotic factors as well as lipid-related factors have been studied and proposed as predictors of CV events. Indeed such hemostatic and thrombotic factors including fibrinogen [134-136], factor VII [137, 138], von Willebrand factor [139], thromboxane [140, 141] and tissue plasminogen activator and plasminogen activator inhibitor-1 [142-144] have been found to be closely related to CVD.

1.1.14. Pharmacological anti-platelet agents: clinical outcomes and consequences

As a consequence of platelet hyperactivity and increased platelet aggregation in the progression of CVD, inhibitors of platelet aggregation are becoming increasingly important in the prevention and treatment of many thrombotic disorders [53]. However, to date, relatively little is known about the direct effects of the most widely used antiplatelet drugs on platelet-dependent activation of coagulation. In addition, the current pharmacological strategies for the inhibition of platelet aggregation, although effective, may present limitations and adverse effects have been reported on cardiovascular health [145-148]. New pharmacological agents, which directly inhibit factor Xa or thrombin, promise efficacy and convenience; however, like warfarin and heparin, they may still compromise hemostasis by causing hemorrhage while preventing thrombosis [149].

It is estimated that approximately 25% of the population that are prescribed COX-1 inhibitors experience some kind of side effect and 5% develop serious health consequences including gastrointestinal bleeding and acute renal failure. A recent review article published in The New England Journal of Medicine reported anti-inflammatory drugs (prescription and over-the-counter, which include Advil®, Motrin®, Aleve®, Ordus®, Aspirin, and others) alone cause over 16,500 deaths and over 103,000 hospitalizations per year in the US [150]. Aspirin has been recommended as the first line drug of choice for primary prevention of CV events, but recent evidence is raising doubt as to its benefit. Without previous disease, aspirin is of uncertain net value as the reduction in occlusive events may not
sufficiently outweigh the increase in bleeding complications [151]. With regards to clopidogrel, no compelling evidence exist that it is a safer therapy than aspirin and therefore it should be reserved to those with aspirin hypersensitivity, intolerance or aspirin resistance. In addition, despite the emerging entity of aspirin resistance [152], aspirin is not an appropriate prophylaxis for VTE [153]. While aspirin plays an important role in the prevention of myocardial and cerebral infarction, uncertainty remains regarding its efficacy in DVT [154]. Furthermore, it has been demonstrated that thrombotic patients taking aspirin for prolonged periods of time develop a progressively reduced sensitivity to the drug [155] and there is limited data addressing the long term safety and efficacy of anti-platelet agents in high risk subgroups such as diabetics [145]. Despite being the most commonly used anti-platelet agent, the mode of action of aspirin on hemostasis has not been fully elucidated. The effects of aspirin were first demonstrated by Szczeklik et al in 1986, reporting the delayed generation of thrombin [156] whereas Reverter et al in 1996 reported only a modest decrease in thrombin generation both in vitro and ex vivo at concentrations that completely inhibited arachidonic acid-triggered aggregation [157]. However, more recent studies observed no significant effect of ingested aspirin (325 mg × 2 /d for 3 days) on TF-induced thrombin generation and even with an addition of 5 μM dipyridamole to whole blood resulted in no effect on platelet-dependent coagulation activity [158]. A similar result was also reported in a much later study by other investigators using a calibrated thrombogram method [159]. It is well known that aspirin inhibits platelet function by irreversible acetylation of a serine at position 529 in the platelet COX [160]. The predominant product of COX-1 in platelets is TXA₂ [36]. Thus aspirin decreases the risk of thrombotic disease in part by blocking the COX-1 enzyme that converts AA to TXA₂, inhibiting platelet aggregation [161]. However, once COX-1 has been acetylated by aspirin, the substrate’s access to its active site is impeded for the lifetime of the platelet. The production of TXA₂ requires the synthesis of new platelets, which are regenerated at a daily rate of approximately 10 percent [162, 163]. Thus the ideal prevention and treatment of thrombotic disease remains an approach that will inhibit thrombosis but unlike aspirin, not compromise hemostasis.

Diet is recognised to play a critical role in modifying an individual’s risk for development and progression of CVD. Natural food products and nutraceutical
agents appear to modify various platelet functions such as cellular adhesion, platelet aggregation and release of procoagulant activity. These include ginger, garlic, tomatoes and long chain omega-3 polyunsaturated fatty acids (LCn-3 PUFA) found in oily fish [164]. Among these, the LCn-3 PUFAs (eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) have gained considerable interest and have accelerated in this regard. As nutrition based on high fish intake has proved to protect against CHD, their cardio-protective effects on platelet function have become a strong clinical focus.

The beneficial effects of consuming LCn-3PUFA from fish and fish oils in reducing the incidence of thrombosis and CVD have been extensively reported [165-167]. Similar to aspirin, the LCn-3PUFAs also interact with COX enzymes. However, unlike aspirin they do not cause irreversible inhibition of platelet aggregation by completely preventing TX formation, rather the LCn-3PUFAs act as a substrate for the enzymes leading to the production of a biologically less active thromboxane moiety - Thromboxane A\(_3\) (TXA\(_3\)) in platelets and an active prostaglandin molecule in endothelial cells [168, 169]. The result is favourably reduced platelet aggregation and increased dilation of blood vessels.

In addition to the generation of TXA\(_3\), theoretically, reduction of platelet aggregation capacity could be achieved by dietary lipid manipulation via reduced release of:

1. platelet-derived microparticles from activated platelet membranes
2. procoagulant agents such as FVa from platelet secretory granules
3. plasminogen activator inhibitor-1 (PAI-1) (enhanced fibrinolysis)

Of utmost importance, most studies that have shown that LCn-3PUFA supplementation or dietary fish intake attenuates platelet aggregation have also reported no observed bleeding episodes [170] or hemorrhagic risk [171] and if such an increased bleeding risk exists, the risk is minimal and not of clinical significance [172]. Moreover, clinical trials have shown high doses of LCn-3PUFA consumption to be safe, even with concurrent administration with other agents that may increase bleeding including aspirin and warfarin [173-175]. Finally, it is important to note that on the basis of existing clinical trial outcomes, the National Heart Foundation of Australia and the American Heart Association have recommended consumption of
1g/day of EPA/DHA in individuals with documented CHD [176]. Thus ideally, supplementation with EPA and DHA in an attempt to minimise cardiovascular episodes should represent a safe and promising prophylaxis for thrombotic disease.

1.2. Long chain omega-3 polyunsaturated fatty acids: A non-pharmacological approach

1.2.1. Lipid Structure and classification
It has been long established that atherosclerosis and thrombosis are both affected by the type and quantity of fatty acids in dietary lipids [177]. Such effects are distinguished by the chain length, degree of unsaturation as well as the position on the triglyceride (TG) backbone, which all contribute to the manner of the digestion and absorption of the fatty acid and how they affect lipoprotein metabolism [178]. Fatty acids form the main constituents of dietary fats and oils including those in contained in plants and animals. They are mainly esterified to glycerol, as triacylglycerols, although some are present as esterified components of phospholipids, glycolipids, and other lipids. Thus they are seldom present as free fatty acids but commonly as ester linked groups within various types of lipid molecules, the triacylglycerols being the most common and of greatest importance [179]. Although dietary fat has been generally considered as deleterious to human health, fatty acids play vital biochemical and physiological roles and some of the most important fatty acids must be obtained from the diet. Amongst these are the essential polyunsaturated fatty acids (PUFAs), the n-6 (linoleic) and the n-3 (α-linolenic) acid, which cannot be synthesised by mammalian cells. In the absence of sufficient dietary intake of these fatty acids deficiency symptoms occur, and hence they are classified as essential fatty acids.

1.2.2. Dietary fatty acids
Fatty acids can affect platelet function by interacting with membrane proteins and serving as precursors for secondary messengers [180]. Their effect depends on the fatty acid chain length and the degree of saturation. Long-chain saturated fatty acids (SFA) have been shown to increase platelet aggregation, whereas intake of short- and
medium-chain fatty acids have been negatively correlated with platelet aggregation [181]. Limited data suggest that monounsaturated fatty acids (MUFAs) may decrease platelet aggregation, however more studies are needed to better understand their effects [182]. A diet rich in MUFA may decrease the prothrombotic environment, by modification of platelet adhesion, coagulation, and fibrinolysis [183]. The effects of polyunsaturated fatty acids (PUFAs) on platelet aggregation have been extensively studied. The two main essential PUFAs are linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3). The major dietary sources of LA are corn and sunflower oils while ALA is found predominantly in green leafy vegetables, flaxseed, canola and soya bean oils. These fatty acids can be further elongated and desaturated to form longer chain n-6 and n-3 long-chain PUFAs, which exert a wide range of biological actions. Few studies have investigated the effects of longer chain-6 long-chain PUFAs on platelet function, with the exception of dihomo γ-linolenic acid (20:3 n-6), which has been shown to activate cAMP formation and inhibit platelet aggregation by competing with AA as a substrate to reduce TXA2 production [180]. In contrast to n-6 PUFAs, the long-chain n-3 PUFAs, particularly from fish oil, have been extensively studied for their potential cardiovascular benefit as a result of reports on the health and diets of Greenland Eskimos [184]. Plasma phospholipid LCn-3PUFAs are a marker of dietary patterns, with higher proportions generally reflecting greater consumption of LCn-3PUFAs and less cardiovascular disease. Contemporary work also suggests that consumption of PUFAs in place of SFA decreases the risk of CVD [185] and in particular EPA and DHA have been associated with measurable improvements in the atherosclerotic profile [186] (or cardiovascular structure and profile). Furthermore both EPA and DHA have a longstanding clinical use for lowering plasma triacylglycerols which are reduced approximately in parallel with the unsaturation index of fatty acids [187].

1.2.3. Omega-3 fatty acid structure and metabolism

The polyunsaturated fatty acids (PUFA) are essential fatty acids that can be divided into the two categories namely the omega-3 (n-3) and the omega-6 (n-6) PUFA. This classification is based on the position of the first double bond from the methyl terminal, being located at the third carbon in the n-3 and at the sixth carbon in the n-6PUFA (Figure 1-6). The n-3PUFA, α-linoleic acid (ALA; 18:3n-3) and the n-6PUFA, linoleic acid (LA; 18:2n-6) are the predominant essential fatty acids and the
precursors of the long chain PUFAs [188]. Both LA and ALA are converted to their long chain metabolites, AA and EPA respectively by the same enzymes however their derived metabolic products from each pathway are structurally and functionally distinct. ALA can be enzymatically converted via desaturation by Δ6 and Δ5 desaturases and elongated to eicosapentaenoic acid (EPA; 20:5n-3). An addition of 2-carbon units to EPA then forms the intermediate docosapentaenoic acid (DPA; 22:5n-3) which is subsequently converted to docosahexaenoic acid (DHA; 22:6n-3) by further chain elongation and Δ6 desaturation; while the n-6 LA is desaturated by the same Δ6 desaturase enzyme and elongated to the n-6 arachidonic acid (AA; 20:4n-6) [189]. The conversion of ALA to EPA and DHA occurs primarily in the liver in the endoplasmic reticulum [190]. It is now also considered that DHA can be biosynthesised by retroconversion via a C24 intermediate followed by beta oxidation in peroxisomes, a pathway known as the Sprecher’s pathway [189] (Figure 1-7). Essentially, EPA is twice elongated yielding 24:5n-3 then desaturated to 24:6n-3 and shorted to DHA (24:5n-3). The ALA-derived EPA is a long-chain PUFA that has 20 carbon atoms and 5 double bonds while DHA has a longer chain, 22 carbon atoms and 6 double bonds. The spatial conformation of DHA is different from that of EPA as a result of its carbon backbone length and degree of unsaturation.
Figure 1-6. Chemical structure of n-3 and n-6 polyunsaturated fatty acids. The n-3 and n-6 PUFAs are classified according to the position of the first double bond at the third and sixth carbon from the methyl end respectively. Adapted from: Cockbain et al (2012) [191].
Alternatively, EPA and DHA can be obtained directly from the diet by consuming oily fish such as salmon, tuna and mackerel. The primary producers of EPA and DHA are algae in the ecosystem and fish consuming algae consequently provide rich sources of EPA and DHA [192]. Obtaining pre-formed EPA and DHA in the diet is essential as the conversion rate from ALA is modest. The metabolic conversion from ALA to the longer chain EPA and DHA vary considerably among species and appear relatively inefficient in humans, partly due to competition with LA for the rate-limiting enzyme, ∆6 desaturase to form AA [193]. As the same enzymes responsible for LCn-3PUFA synthesis are also involved in the conversion of the n-6 LA to AA, dietary background is an influential factor in the conversion rates. Consumption of LA in a typical western diet is approximately 10 times greater than ALA [194] suggesting the metabolism of the former will predominate. Indeed diets high in LA have been reported to reduce conversion of ALA to EPA and DHA by 40% due to substrate competition and inhibition of ∆6 desaturase enzyme. However, considerable variability in the conversion rates has also been reported within subjects with similar background diets [195].

In particular for males, the conversion to EPA is limited with further transformation to DHA being very low or absent. The fractional conversion of ALA to EPA varies between 0.3% and 8% and from ALA to DHA is < 4% or absent in men [196-199]. Comparatively, conversion of ALA to EPA and DHA is greater in females due to a regulatory effect of oestrogen. From ALA, up to 21% and 9% is converted to EPA and DHA respectively. This capacity to up-regulate ALA conversion may be important for meeting the demands of the foetus and neonate for DHA [200, 201]. Furthermore, this supports that variations in metabolic capacity for ALA desaturation and elongation may be due in part to hormonal influences rather than diet alone. In response to pre-formed EPA supplementation, plasma concentrations of EPA but not DHA significantly increase [202-204]. However, DHA supplementation results in both a dose-dependent saturable increase in plasma DHA [205], and a linear rise in plasma EPA concentrations via retroconversion through a β-oxidation reaction [206]. Retroconversion from DHA to EPA in humans receiving normal dietary amounts of DHA has been calculated to be between ~1.4 - 12% [207-209]. Plasma EPA concentrations increase by approximately 0.4g/100 g fatty acid for 1g of DHA intake. Additionally, retroconversion from DHA to EPA is hormonally regulated and
decreases in women receiving HRT [210]. These retroconversions are of importance due to the formation of C20 fatty acids which are incorporated into PL and subsequently act as a substrate for eicosanoid formation.

Figure 1-7. Synthesis of n-3 and n-6 polyunsaturated fatty acids to their longer chain derivatives. EPA and DHA compete with AA for the Δ 5 desaturase enzyme for prostaglandin and leukotriene synthesis to produce eicosanoids of 3 and 2 series respectively. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Adapted from: Curtis et al (2004) [211].
1.2.4. Dietary recommendations

Epidemiological studies indicate that humans evolved on a diet with a ratio of n-6: n-3 PUFA of approximately 1, compared to the modern typical western diet where the ratio has increased to 10-30:1 [212, 213]. This change has been attributed to the increased intake of n-6PUFA coupled with a decreased intake of n-3PUFA, particularly EPA and DHA. In the western diet, AA is the most abundant 20 carbon PUFA available as a substrate for eicosanoid synthesis due to the greater abundance of LA over ALA and in addition preformed AA from meat as opposed to its n-3 analogue EPA from fish. Consequently, the tissue PLs are enriched in AA. Unfortunately, a high n-6: n-3 ratio promotes the pathogenesis of many diseases, including CVD, cancer and inflammatory diseases, whereas increased levels of LCn-3PUFA (lower n6:n3), with an optimal ratio of 2-4:1 exert suppressive effects due to eicosanoid function [214]. For CVD prevention, the National Heart Foundation (NHF) of Australia and the American Heart Association (AHA) recommend 2-3 servings of oily fish a week or 500mg/day of EPA/DHA for adults [176]. However, the Australian National Health and Medical Research Council (NHMRC) estimate that consuming at least 160 mg/day for males and 90mg/day for females is adequate. While individuals with documented CHD or with lipid abnormalities are recommended by the AHA to consume 1000mg/day of EPA/DHA [176].

1.2.5. Dietary fatty acids in cell membranes

Mammalian cell membranes consist of a lipid bilayer composed primarily of PL and cholesterol. Proteins that drive critical cellular functions, such as receptors, transporters, and enzymes are embedded in the lipid bilayer [215, 216]. Compositional FA variations and interactions in the lipid bilayer can be of sufficient magnitude to alter membrane physical properties and affect certain membrane functions. In cell membranes, FAs can be found either as constituent of membrane PL (i.e., esterified FAs) or as free molecules (i.e., free FA — FFA). In both forms, LCn-3PUFA have a remarkable contribution to the physical properties of biological membranes and the ability to modulate the activity of membrane-associated proteins [217]. The same types of membrane FA variations that can be produced in cultured cells occur in animal models when the dietary fat intake is modified [215, 218, 219]. In humans, membrane FA alterations also have been produced in erythrocytes and platelets by dietary fat modifications [220]. Polyunsaturated dietary fat intake alters
the FA composition of cellular membranes and plasma lipoproteins with a resultant increase in fluidity. Fatty acid alterations in the membrane PLs may induce subtle changes both in the inner and the outer plasma membrane. The membrane lipid composition is highly important given that the outer platelet membrane leaflet provides the catalytic surface for expression of platelet receptors and subsequent adhesion and aggregation of platelets. The major unsaturated LCFAs incorporated into cell membranes of humans are the n-3 EPA and DHA; n-6 AA and the n-9 oleic acid [33]. These fatty acids are released from the PLs under a variety of stimuli to undergo enzymatic degradation to the eicosanoids. Indeed, the most favourable functional effects have resulted from LCn-3PUFA enrichment; altering prostaglandin formation, decreasing platelet aggregation and blood coagulation proteins [221-223].

1.2.6. Eicosanoid formation

The eicosanoids are derived from 20-carbon polyunsaturated fatty acids which are present as components of cell membrane phospholipids. Activation of phospholipases causes release of these fatty acids that can be metabolized either via the COX pathway to produce the prostanoids - prostaglandins (PG), thromboxanes (TX), and prostacyclins (PGI) - or via the lipoxygenase (LOX) pathway to form leukotrienes (LT) and lipoxins. These fatty acids can also be oxidized by the cytochrome P-450 system giving rise to several metabolites [224]. The metabolites derived from the n-3 EPA and n-6 AA are eicosanoids which have profound effects on biological responses and are responsible for many of the effects found in inflammatory conditions. The beneficial effects the LCn-3PUFAs have been widely discussed with regards to AA metabolism in platelets and the vessel wall [225, 226]. In general, eicosanoids derived from AA are pro-inflammatory and pro-aggregatory agonists while those derived from EPA are anti-inflammatory and reduce platelet aggregation [227]. EPA and DHA compete with AA for prostaglandin and leukotriene synthesis at the COX and LOX level to produce eicosanoids of 3-series (PGE₃, PGI₃, TXA₃ and LTB₅) or 2-series (PGE₂, PGI₂, TXA₂ and LTB₄) respectively [228]. Though ALA suppresses AA levels and consequently the eicosanoids derived from it, it is not a substrate for LT biosynthesis. Conversely, oleic acid is not a substrate for PG synthesis but can form LTs. While AA, EPA and DHA are major substrates for eicosanoid formation, DHA is also a potent inhibitor of COX but not of LOX in vitro [33]. The TXs and PGs play an important role in the
balance between hemostasis and thrombosis. The underlying cardio protective effects of EPA and DHA reside in their ability to compete with AA for PG, TX and LT synthesis to modulate the production of pro-thrombotic eicosanoids [228].

Platelet aggregation in primary hemostasis is initiated by the release of AA from membrane phospholipids resulting in generation of AA derived eicosanoids TXA\textsubscript{2} and PGI\textsubscript{2} [167]. TXA\textsubscript{2} is a potent aggregatory substance synthesised via COX pathway while PGI\textsubscript{2} has opposing effects and is synthesised via the LOX pathway in the vascular endothelium. In contrast, the eicosanoids derived from EPA; TXA\textsubscript{3} and PGI\textsubscript{3} have opposing effects and act as weaker stimuli for platelet aggregation and vasoconstriction. Incorporation of LCn-3 PUFAs into platelet PL displaces AA from the platelet membranes to compete as a substrate for the COX enzyme and catalyses the biosynthesis of TXA\textsubscript{3} and subsequently reduced conversion to TXA\textsubscript{2} [167, 229-231]. TXA\textsubscript{3} derived from EPA is biologically less active compared to TXA\textsubscript{2} while the anti-aggregatory effects of PGI\textsubscript{2} and PGI\textsubscript{3} remain comparable resulting in a favourable PG\textsubscript{2}/TXA\textsubscript{3} balance [232]. Though DHA is not a direct substrate of COX, and can in fact act as an inhibitor of COX \textit{in vitro}, however DHA and EPA can be interconverted to each other (Figure 2-7). As previously described, DHA supplementation results in both a dose-dependent saturable manner in plasma DHA [205], and a linear increase in plasma EPA concentrations via retroconversion through a \(\beta\)-oxidation reaction [206]. Indeed administration of fish oils containing both EPA and DHA favourably increases the n-3/n-6 PUFA ratio in the membrane PLs, the sum of PGI\textsubscript{2} and PGI\textsubscript{3}, and effectively reduces TXA\textsubscript{2} production in human studies and animal experiments \textit{in vivo} [225, 233]. Furthermore, DHA has been also found to antagonize prostanoid TP receptors in platelets and aorta [225]. Thus the \textit{in vivo} increase in PGI\textsubscript{2}/PGI\textsubscript{3} after n-3 PUFA supplementation may be explained, at least in part, by the retroconversion of DHA to EPA. It follows that a dietary imbalance in favour of n-6 AA may have detrimental effects on cardiovascular health and can be a significant factor contributing to the rising rate of CVD [234]. As the required amounts of EPA and DHA cannot be sufficiently synthesised from ALA, obtaining pre-formed EPA and DHA directly from the diet in the form of fish or fish oil supplements is important for adequate incorporation into cell membranes and uptake in body tissues [201].
1.2.7. Epidemiological, observational and prospective studies

The observation that LCn-3PUFA may exert beneficial effects on ischemic heart disease, cerebral and myocardial infarction (MI) has been delineated from a plethora of scientific reports in the literature. In the 1970s, Dyerberg and Bang conducted a series of prospective studies on Greenland Eskimos [184, 235-239] and revealed that the rarity of ischemic heart disease and decreased thrombotic tendency in this population was linked to their consumption of a seafood diet high in LCn-3PUFA. These findings contrasted sharply when compared to the dietary habits of an ethically similar population in Denmark with significantly higher rates of CVD [240]. The diet of the Danes had a comparable amount of total fat, a higher intake of saturated fat but a much lower intake of LCn-3PUFA, whereas Greenland Eskimos consumed more LCn-3PUFA at the expense of saturated fat [184]. The Greenland Eskimos also exhibited lower cholesterol levels due to lower concentrations of LDL and VLDL [240] and significantly longer bleeding times compared to the Danes; the prolonged bleeding time due to decreased platelet aggregation was accompanied by a shift from the n-6 to LCn-3PUFA in platelet fatty acid composition [223]. Thus Dyerberg et al. suggested through their landmark studies that EPA may potentially inhibit thrombosis and atherosclerosis [241].

The same phenomenon of lower CVD rates was later reported in other populations consuming a high seafood diet including Alaska [242], Japan [243] and China [244]. In the Japanese male cohort, those that consumed more fish (fisherman) also had a much lower blood pressure as well as incidence of coronary artery disease (CAD) compared to the Japanese farmers that consumed less fish [245]. Observational studies in the Western population reported an inverse relationship between fish consumption and coronary heart disease (CHD) mortality in the Rotterdam [246] and the Zutphen study [247] in the Netherlands and in a cohort of the Chicago Western Electric study [248] in the US. The Zutphen study reported an inverse dose relationship between fish consumption and CHD mortality in men where higher fish consumption at baseline with the subsequent 20 year follow of CHD mortality was 50 % lower in men who consumed more than 30 g of fish per day compared to those that did not consume fish [247]. Similar findings were reported in the Western Electric study with a 30 year follow up of CHD risk in men; fatal myocardial infarction rates were significantly lower in those who ate ≥ 35 g fish daily than those
that did not [248]. Notably, these early observational studies were conducted in male populations.

In the Cardiovascular Health Study, Mozaffarian et al reported in a cohort of 3,910 men and women that consumption of tuna, broiled or baked fish but not fried fish was associated with lower risk of fatal CHD and arrhythmic death [249]. This was of importance given that associations varied depending on the type of fish meal consumed, as EPA/DHA composition in fish can also vary comparatively. Tuna consumption was associated with a more favourable cardiovascular risk profile which was also associated with younger age and female gender. The heterogeneity of LCn-3PUFA content in different fish types in relation to plasma EPA and DHA concentrations has been demonstrated by Chung et al [250]. This cross-sectional study corroborated the results by Mozaffarian et al [249] indicating that fried fish serves as a poor source of LCn-3PUFA and further demonstrated that the type of seafood consumed influences plasma EPA and DHA concentrations. However, both these studies did not run correlations between the varying EPA or DHA compositions with gender, or discriminate between the differential EPA or DHA content. Though the former was of provocative interest paving the way into gender influences given that the cardiac benefits of fish consumption varied depending on gender and the type of fish meal consumed independently.

Other studies which have included a wider scope of variables have shown improved correlations with hemostatic variables. The Northwick Park Heart Study (NPHS) investigated the thrombotic component of ischaemic heart disease (IHD) among 1511 Caucasian males and observed that high levels of FVII coagulant activity and plasma fibrinogen were associated with coronary events more so than cholesterol [251]. The Caerphilly and Speedwell Collaborative Heart Disease Studies reported that fibrinogen, plasma viscosity and white blood cell count were important risk factors for CHD; when added to a model containing the major conventional risk factors, there was a highly significant improvement in the fit of the model [252].

In the pathogenesis of cardiovascular events, inflammation also plays an important role with the recognition that atherosclerosis is an inflammatory process [132]. Several epidemiological studies have indicated the beneficial effects of LCn-3PUFA
on inflammation and endothelial function. Inverse associations have been found between the intake of LCn-3PUFA and plasma concentrations of biomarkers of inflammation and endothelial activation, including C-reactive protein (CRP), tumor necrosis factor α (TNFα), E-selectin and intercellular adhesion molecule-1 (ICAM-1) [253-255]. The association between fish intake and atherosclerosis have also been examined in observational studies and reported that serum LCn-3PUFA levels were inversely related to the probability of carotid plaques [256] and significantly less progression of coronary atherosclerosis [257].

An ecological study conducted by Zhang et al [258] included 36 countries and examined the relation between fish consumption and all causes of mortality, ischemic heart disease (IHD) and stroke, where data was obtained from the Food and Agricultural Organization and the World Health Organization. Significant, independent inverse correlations were found between fish consumption and all assessed factors, even after exclusion of countries with the highest amount of fish consumption and lowest all-cause mortality rate. The statistical analyses in the study demonstrated that fish consumption is associated with the reduced risk of death from all causes, IHD and stroke mortality at the population level in a diverse number of populations.

Though several studies have reported beneficial effects of fish consumption on CHD, this has not been a consistent finding in all epidemiological studies. Earlier trials investigated the effects of LCn-3PUFA in the secondary prevention of MI. In the Diet and Reinfarction Trial (DART), 2,033 male patients recovering from MI were randomly allocated dietary advice to either reduce the ratio of polyunsaturated to saturated fat, increase in fatty fish intake or increase in cereal fiber intake. Patients who were advised to increase their fish to at least 2 fish meals a week had a 29% decrease in 2 year all-cause mortality rate compared to those not advised but with no decrease in the rate of non-fatal MI [259]. Conversely, the number of nonfatal MIs tended to increase rather than decrease, which made the underlying mechanism of protection in the fish advice group unlikely to be anti-thrombotic.

Similar findings were reported in a cohort of 1,847 men from the Chicago Western Electric Study where consumption of fish from none to more than 35g/day had
revealed no association with stroke incidence [260]. Interesting, stroke rates were highest in the subgroup reporting the highest fish intake; the authors reported no inverse association of fish consumption with stroke but rather a potential adverse effect. Contrasting results were reported in the GISSI-Prevenzione trial involving 11,321 primarily male MI patients. Recent post MI patients were randomly assigned to supplements of LCn-3PUFA (1g/day), vitamin E (300 mg/day), both or none for 3.5 years. Patients receiving treatment of n-3 FA experienced significantly lowered combined risk of mortality, non-fatal MI and stroke by 10-15% compared to no LCn-3PUFA. No effect was shown for vitamin E [261].

A meta-analysis including 10 trials reviewed the effects of LCn-3PUFA on cardiovascular events in CHD patients. Daily supplementation with LCn-3PUFA decreased the incidence of all-cause mortality by 16% and decreased the incidence of fatal myocardial infarction by 24% but no significant effects were found for non-fatal MI, non-fatal stroke and angina [262]. These studies suggested the anti-arrhythmic effects from LCn-3PUFA intake or fatty fish (2 serves per week) may reduce mortality in recent post MI and CHD patients. However other studies have also reported unfavorable results. The post-trial follow-up data of the DART trial showed no long term survival benefit in the post MI patients. The early reduction in mortality observed in those patients given dietary fish advice over the 2 years of the trial was conversely followed by an increased risk (hazard ratio 1.31) over the next 3 years [263]. Another study also reported that advice to eat 2 portions of fatty fish per week or 3g fish oil daily was associated with higher risk of cardiac (hazard ratio = 1.26) and sudden death (hazard ratio = 1.54) in patients with angina over a period of 3-9 years [264].

The US Physicians’ Health Study examining a cohort of 21,185 male physicians after 4 years follow-up reported no association between dietary fish intake and any cardiovascular endpoint, including MI, stroke and cardiovascular death [265] even after adjustment for cardiovascular risk factors. However, it was later observed in 20,551 male physicians followed over 11 years that sudden cardiac death was reduced in men that consumed at least 1 fish meal per week even after controlling for aspirin use, but without changes on fatal MI [266]. This prospective study also demonstrated a threshold effect at a consumption level of 1 fish meal per week.
Furthermore, the Health Professional Follow-up Study of 43,671 males over 12 years reported that the risk of ischemic stroke was significantly lowered in men that consumed fish 1-3 times per month compared to those that consumed less than 1 fish meal per month. However, no association between fish oil intake and risk of stroke was evident, though the number of men taking fish oil supplements was small. It is interesting that the beneficial effects of such a small amount of fish intake were associated with a significantly lower risk of ischemic stroke however lacking a dose response effect. However, this is in line with the threshold effect proposed by Albert el al in The US Physicians’ Health Study, no correlations were observed with the number of fish servings per month in this study, and this was not influenced by the use of aspirin [267]. Since both aspirin and LCn-3PUFA inhibit platelet aggregation through the inhibition of TXA₂ synthesis, this may suggest that fish intake may not provide any additional benefit among men using aspirin.

These contrasting studies were conducted in males, most without use of repeated measures of fish intake in the analysis, few adjusted for potential confounding dietary variables and without examining the risk with specific stroke subtypes. These are accountable factors given the contradictory reports and the early observations that fish consumption reduces the risk of ischemic stroke [268] but may increase the risk of haemorrhagic stroke [269] as suggested initially by the very high LCn-3PUFA diet of the Greenland Eskimos.

In 2001, Iso et al examined the association between LCn-3PUFA intakes with the risk of stroke subtypes in women in a cohort from the Nurse’s Health Study comprised of 79,839 females nurses followed up over 14 years. Among stroke subtypes, a significantly reduced risk of thrombotic infarction was observed among women with consumption of 2 or more fish meals per week and women in the highest quintile of LCn-3PUFA intake had reduced risk of total stroke and thrombotic infarction. When stratified by aspirin use, LCn-3PUFA intakes were inversely associated with risk of thrombotic infarction, primarily among women who were not regular aspirin users. Most importantly, no association was observed between LCn-3PUFA intake and risk of haemorrhagic stroke [270].
Further acknowledging the limited data with respect to females, a later study comprised of 84,688 females also from the Nurse’s Health Study examined the association between fish and n-3FA intake and incidence of CHD among women. This study further adjusted for potential dietary and lifestyle confounding variables followed up over 16 years. A higher risk of CHD was observed in women that consumed less than 1 fish meal per month compared to those with higher intakes [271]. Furthermore, the lower risk of CHD death was associated with higher intakes of n-3 FA irrespective of regular aspirin use. In contrast, the inverse association between n-3FAs and nonfatal MI was significant only among women who did not use aspirin regularly. The women that consumed more fish were associated with a healthier diet and lifestyle. However, adjustments were made for potential dietary and lifestyle confounding variables and these did not appreciably alter the results, suggesting an independent effect of fish and omega-3 fatty acids on CHD risk. More specifically, these associations were independent of established cardiovascular risk factors and dietary predictors of CHD such as fibre, trans-fatty acids, and the ratio of polyunsaturated to saturated fats or by variations in intake of red meat, fruits or vegetables.

1.2.8. The problem: a controversy
Although substantial evidence in the literature largely suggest that LCn-3PUFA may improve cardiovascular health through a wide-range of biological effects including altering lipid metabolism [272], hypertension [273, 274], modulating platelet function and coagulation proteins [275, 276], improving endothelial function and inhibiting inflammatory pathways [277], several epidemiological studies have also reported equivocal results for protection against vascular occlusions [261, 265]. Such studies have pointed at an effect of LCn-3 PUFA on fatal CHD and sudden cardiac death rather than an effect on nonfatal MI suggesting that an antithrombotic effect of LCn-3 PUFA was not the major reason for the observed effect. These unexpected, contradictory findings challenge the previous beneficial results of fish consumption against thrombosis; weakening the suggestion of an anti-thrombotic effect of LCn-3 PUFA.

The inverse relationship between fish intake and risk of stroke and thrombotic occlusions have been reported in several [278, 279] but not all [260, 265] prospective
studies. Few observational studies have adequately considered the types of seafood and background diet when evaluating diet-biomarker and diet-disease associations. This may partly account for inconsistent findings in observational studies on cardiovascular health effects of LCn-3PUFA or fish consumption. In addition, these contrasting studies were conducted in males, most without use of repeated measures of fish intake in the analyses, few adjusted for potential confounding dietary variables and none with gender-specific analyses or examination of EPA versus DHA.

Furthermore, several conflicting results from dietary intervention trials have been reported against platelet reactivity. Many trials have studied the effect of LCn-3 PUFA on platelet function, coagulation and fibrinolysis in healthy humans and in patients with or at increased risk of CHD. LCn-3 PUFA has had inconsistent effects on measures of coagulability or fibrinolysis. These unexpected, contradictory findings challenge the previous beneficial results of fish consumption against thrombosis; weakening the suggestion of an antithrombotic effect of LCn-3 PUFA. Consequently, the anti-aggregatory effects of LCn-3PUFA have become controversial. Despite such scientific effort and decades of clinical studies, the precise mechanistic pathways involved in the anti-thrombotic effects of LCn-3 PUFA remain complex and unclear. In addition, studies have generally used combinations of EPA and DHA, making it difficult to discern specific roles and health benefits of the individual LCn-3PUFAs.

1.2.9. The resolution: a controlled approach

Ongoing human clinical trials involving omega-3 fatty acid supplementation continue to yield equivocal evidence for their effectiveness against platelet aggregation reporting conflicting results [280-285]. It may be possible that limitations in the efficacy of LCn-3PUFA supplementation may be partly inherent in their mechanism of action that is dependent on subject biochemistry (eg. hematological parameters, sex hormonal levels) therefore should not be deemed ineffective or disregarded. Individual variability of response to LCn-3PUFA plays a substantial role. With respect to platelet function, variability of response may result from baseline and residual platelet characteristics, increased platelet turnover, vascular function and lipid metabolism; all of which are physiological non-
modifiable factors and attributable to the role of gender. In addition, fish oils (FO) are a heterogeneous product and despite the fact that fish and FO supplementation contain both EPA and DHA and at variable amounts, relatively few studies have attempted to discriminate between their effects on platelet reactivity. Commercially available FO capsules contain variable mixtures of EPA and DHA and most present a proportion of 2:1 for EPA and DHA respectively. However, whether the anti-thrombotic effects of FO supplementation are attributed to EPA, DHA or both has not been distinguished.

With respect to diet, fish can be classified into lean fish that store lipid as triacylglycerols in the liver (e.g. cod) or fatty/oily fish that store lipid as triacylglycerols in the flesh (e.g. mackerel, salmon, tuna) [286]. It should also be noted that different types of fish contain different amounts of LCn-3PUFA. This is partly dependent upon the metabolic characteristics of the fish and also upon their diet and external factors such as water temperature and season. Recent studies investigating the effect of changing the type of dietary oil fed to farmed salmon have reported significant effects of this on LCn-3PUFA content of the flesh [287, 288]. Fish oil that is obtained from lean fish livers or fatty fish flesh has the distinctive characteristic of being rich in LCn-3PUFAs; thus given that different oily fish contain different amounts of LCn-3PUFAs, so do fish oils. In addition, it is not only the amount of LCn-3PUFAs that can vary between fish and fish oils, but also the relative proportions of the individual LCn-3PUFAs (EPA, DPA and DHA).

Furthermore, until 1990, purified DHA was not available and there was no study regarding its effects in humans, consequently most of the reported studies were performed with FOs containing higher proportions of EPA than DHA. Concomitantly, the majority of studies that were conducted were on males or unequal gender groups. The traditional model of clinical research was limited by gender until 1993 when the National Institutes of Health mandated enrolment of women in human clinical trials [289]. Historically, women have been excluded from landmark research studies including early reports on the anti-thrombotic effects of LCn-3PUFA [221, 290-297]. It should not be assumed that results of research on male participants could be easily extrapolated to female populations; while gender-specific pharmacodynamic data are meagre, evidence also supports the existence of sex-
related outcomes [298]. Underrepresentation of females compromises and undermines the clinical utility of research results. In the available fish oil and platelet aggregation literature, wide variability in terms of dosage, study design and gender inequality are apparent, hence there is discrepancy regarding the effect of fish oils on platelet activity in human subjects. Disparate findings concerning effects of LCn-3PUFA on platelet function may be explained by gender-specific differences in platelet aggregation in response to EPA or DHA.

Irrespective of the bias caused by heterogeneity of the fish oil dosage, gender itself is an independent predictor of CVD outcome and thus represents a confounding factor in such studies [299]. The available published literature and studies to date have been conducted either in men, unequal gender groups or in patients with cardiovascular complications. Observational clinical studies in this area have either compared the sex hormone profiles of men and women with and without coronary disease or computed the relative prevalence of disease in populations that differ in their sex hormone patterns. Furthermore, there is wide variability in terms of dosage, study design and subject characteristics. Hence, there is discrepancy regarding the effect of fish oils on platelet activity in human subject. The individual assessment of EPA versus DHA in addition with sex-specific analyses may likely explain the inconsistent results in the fish oil and platelet aggregation literature.

1.3. Gender influences in platelet aggregation

1.3.1. Gender differences in thrombosis
Cardiovascular gender differences are apparent long before CVDs appear and many of these differences may be attributed to the development of thromboembolism and CVD. Despite these several differences [300, 301], the majority of studies have continually ignored gender as a confounding factor. Sex is an independent risk factor for several thrombotic processes including MI, VTE and thrombotic stroke [302, 303]. Over the last decade, compelling evidence has supported the idea that the dissimilar impact of CVD is attributed to the cardiovascular and metabolic effects of sex steroid hormones [299, 304, 305]. Interestingly, the majority of hemostatic
markers have been reported to be correlated or influenced by sex hormonal levels [306] and accordingly a male to female ratio of CVD of 2:1 has been a consistent finding [307]. This differential persists even when the classic risk factors for CVD including, hypertension, smoking, obesity, diabetes, and hyperlipidemia are controlled for gender. The most likely ultimate cause of this phenomenon is male to female differences in sex hormone patterns. Indeed, androgens and oestrogens influence a multitude of vascular biological processes and their cardiovascular effects are multifaceted. Such differences in thrombosis are well described; men are 50% more at risk to suffer recurrent VTE than pre-menopausal women [303, 308], however there is a menopause-associated increase in CVD thereafter, in addition women on HRT have a 2- to 5-fold increased risk of venous thrombosis compared with nonusers [309].

Moreover, inverse relationships between serum testosterone and the degree of atherosclerosis have been reported [310, 311], thus it is speculated that hemostatic mechanistic responses may differ in accordance with varying sex hormonal concentrations. Regrettably, females have generally been either excluded or underrepresented in cardiovascular trials and consequently the evidence base is rather unsatisfactorily extrapolated from observational cohorts or from small numbers within larger randomised trials. Emerging data indicate that men and women do not accrue equal benefit from antithrombotic therapy [312, 313]. Thus there is a pressing need to ensure that cardiovascular trials are specifically designed to incorporate sufficient numbers of females to allow gender-specific efficacy analyses to be undertaken.

### 1.3.2 Cardiovascular and hemostatic gender differences

Given that risk factors may differ in impact according to gender, most cardiovascular events could have been prevented in both sexes with a tailored approach. Sex hormones are potent modulators of cardiac risk factors at each level of the atherosclerotic process as they alter procoagulant protein expression and the function of blood and vascular cells [314, 315]. Sex-specific physiological differences that may influence cardiovascular outcome include lower BMI and smaller organ size in females compared to males resulting in larger distribution volumes in men. Furthermore a higher proportion of body fat in females may increase the distribution
volume for lipophilic substances [316]. In addition, females have higher platelet counts, greater baseline platelet aggregation [317-319] and their platelets are also reported to be intrinsically more sensitive to agonist than male platelets [320]. Due to hormonal differences, females have been reported to have more active platelets than males [305]. Indeed variations in circulating concentrations of estrogens and/or progestins have been suggested to modulate GPIIb/IIIa function for platelet activation. Analysis of data from female subgroups demonstrated an association of GPIIb/IIIa reactivity with menstrual phase where the number of GPIIb/IIIa receptors per platelet capable of binding fibrinogen was significantly greater in premenopausal women than men [317].

Female sex and oral contraception, are associated with elevated fibrinogen levels [134] while accordingly is inversely related with testosterone levels [321]. Moreover, testosterone administration causes a significant fall in fibrinogen [322] while postmenopausal women and low serum testosterone in men is associated with high factor VII levels [323, 324]. Declines in oestrogen at menopause are associated with increased numbers of procoagulant platelet-derived MP [131] and this increase is suggested to be modulated by the menstrual cycle [315]. Oestrogen levels have been reported to be inversely associated with P-selectin [325] and females have significantly lower levels of CAM expression [253]. The positive association between testosterone levels and tPA (stimulator of thrombolysis) and a negative association with coagulation factor VIIa and PAI-1 (inhibitor of thrombolysis) has been a consistent finding [326-328]. PAI-1 plays an important role in coronary disease since elevated levels predict MI and progression of atheroma in stable CAD patients [144, 329]. Indeed replacement of testosterone in hypogonadal men with elevated PAI-1 levels and therapy in normal men has led to reductions in circulating PAI-1 levels [130, 330].

Several studies have also reported that testosterone regulates the expression of platelet TXA<sub>2</sub> receptors, thereby increasing vascular responses to TXA<sub>2</sub> [331, 332]. It has been demonstrated in healthy males that administration of testosterone increased platelet TXA<sub>2</sub> receptor density and aggregation responses [333]. Compelling data have indicated that sex differences in vascular biology are determined not only by sex-related differences in sex hormonal levels, but also by sex-specific tissue and
cellular differences that mediate sex-specific responses [304]. The majority of these hemostatic markers have been reported to be correlated or influenced by sex hormone levels [334, 335] while sex hormone levels in turn influence LCn-3PUFA uptake and metabolism [200, 336-339].

1.3.3. Gender differences in blood LCn-3PUFA content and metabolism
The LCn-3PUFA content in tissues is important in maintaining a balanced lipid profile as lipid abnormalities contribute substantially to the atherosclerotic process [340]. Indeed higher plasma concentrations of LCn-3PUFA are associated with a lower risk of MI [341] and thrombotic disease [342]. In particular, a low EPA+DHA level appears as a risk for sudden cardiac death where a markedly reduced risk of sudden cardiac death was observed in patients that exhibited 3% higher EPA+DHA levels compared to those in the quartile with the lowest EPA+DHA levels [343]. Dietary-induced changes in fatty acid composition of human plasma and platelets follow a similar time course and is a major determinant of their membrane fatty acid composition [344].

Interestingly, in men, plasma phospholipid EPA but not DHA was reported to be inversely associated with carotid intima-media thickness, a surrogate measure for sub-clinical atherosclerosis [345]; while higher plasma DHA levels in serum phospholipid have been associated with reduced progression of coronary atherosclerosis in women [346]. Di Stasi and Bernasconi conducted a study to investigate the time course and extent of incorporation of n-3 PUFA in plasma and platelets with 1g/day of n-3 PUFA for 12 weeks. The study demonstrated that 1g/day of n-3PUFA was able to induce striking changes within a week of supplementation with incorporation into plasma PL and platelets by 62% and 27% respectively. EPA in plasma PL increased 289% where the increase of DHA (23%) was not significant. Of interest, the cohort was comprised of male subjects only and among the individual LCn-3PUFAs, the raise of DHA was the least efficient in plasma PL and platelets [32]. Silverman and colleagues were able to demonstrate effects on platelet aggregation after a single dose of n-3PUFAs given as fish or fish oil [347]. Similarly, absorption of EPA was more efficient in plasma and platelet aggregation was significantly reduced [347]. It is unfortunate that the investigators did not report the sex of the study subjects. Regardless, the investigators demonstrated that a single
dose of n-3PUFAs can induce immediate effects to substantially reduce platelet aggregation. This is of significance as it highlights the immediate effects by a mechanism not requiring incorporation into platelet membranes but within the circulating plasma.

More recently, distribution, interconversion, and dose response of n–3 fatty acids in humans have been suggested to be influenced by gender [206]. Biosynthesis of LCn-3PUFAs are different in men and women [200] and a number of studies have reported gender differences in circulating plasma concentrations of LCn-3PUFA. Data from studies using stable-isotope-labelled ALA gave the first indications of gender differences in the ability to synthesise LC n-3 PUFA from ALA [188]. Females have higher concentrations of DHA and lower concentrations of EPA and DPA compared with males and the difference is independent of dietary intake [336-339] indicating either a greater rate of conversion to DHA of their incorporation into membrane PL. In females, the synthesis pathway for DHA is more responsive to the dietary availability of LCn-3PUFA. It was demonstrated in females that received a beef-based diet were able to utilise a 3-fold greater amount of DPA to generate DHA compared with males [348]. The ability of men to generate DHA from DPA was not affected by diet. In a male cohort, a diet high in ALA did not affect conversion to EPA or DHA but an EPA/DHA enriched diet decreased ALA conversion to EPA but not DHA [349]. In addition, EPA is also more readily mobilized into platelet phospholipids 3-4 fold higher than DHA and DPA in males [350]. Collectively, these studies indicate that background diet may interact with gender difference in the metabolic conversion of LCn-3PUFA. Furthermore, gender differences exist in the rates of β-oxidation and potential influences of sex hormones on the desaturase and elongase enzymes responsible in the synthesis of LCn-3PUFA have been proposed [200, 351]. Although limited information is available on this precise mechanism, it has been shown in an animal model that oestrogens enhance the expression of genes involved in FA β-oxidation through PPAR-mediated peroxisomal proliferation [141]. Therefore the final step in DHA synthesis may be modulated differently between the genders due to hormonal control. The role of sex hormones in mediating the gender differences reported in ALA conversion and LCn-3PUFA concentrations have been suggested from a number of females studies using oral contraceptives [200, 204, 337], or hormone replacement therapy in women [352, 353] and in a study involving
sex-hormone treatment in transsexual subjects where an increase in DHA status was observed with oestrogen administration while in contrast, decreased with testosterone [337]. Furthermore, in males retroconversion of DHA to EPA has been described [205]. Thus not only dietary intake, but gender differences in lipid absorption and metabolism are important factors that affect the fatty acid profile of circulating lipids. Furthermore, this may indicate important risk differences between males and females based on metabolism of the LCn-3PUFAs which may affect the responsive to supplementation. With respect to platelet activity, the relative proportion of these fatty acids may determine their availability as substrates for cyclo-oxygenases, hence the influencing the balance of eicosanoids [354] as well as effects on the coagulant potential.

1.3.4. Conflict: EPA and DHA on platelet activity and coagulation

It is becoming increasingly evident that primary hemostasis through platelet activation and aggregation and secondary hemostasis through clotting are processes that amplify each other in all types of hemostatic and thrombotic events. Mechanisms for protection against vascular occlusions by LCn-3PUFA intake may include inhibition of platelet aggregation, including modification of the eicosanoid system (eg, reducing the production of TXA₂), alteration of the fatty acid composition of membrane phospholipids, decreased procoagulant activity, reduction of coagulation factors and CAM, as well as enhanced fibrinolysis. Many studies report an effect of LCn3-PUFA on hemostasis however with high inter-study variation. While some have demonstrated reductions in platelet activity, coagulation and fibrinolysis, others on the contrary do not. Table 1-3 summarises and compares 21 studies reporting such variable results.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects</th>
<th>M:F ratio</th>
<th>Intervention</th>
<th>Follow-up</th>
<th>Platelet aggregation</th>
<th>TX</th>
<th>Hemostatic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mori et al [275]</td>
<td>120 HL, HT subjects 1:0</td>
<td>Fish oil 3.5g/d (2.2g EPA/1.4g DHA) vs. Placebo (olive/palm/safflower oils)</td>
<td>12 weeks</td>
<td>↓ (~7%) collagen- and (~9%) PAF-induced with fish oil</td>
<td>↓ (~10%) TX with fish oil (collagen-induced)</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Agren et al [355]</td>
<td>55 HS 1:0</td>
<td>DHA-oil (1.68g/d) vs. Fish diet (1.05g/d EPA+DHA) vs. Fish oil (2.28g/d EPA+DHA)</td>
<td>15 weeks</td>
<td>↓(-36%) collagen-induced with fish diet and (-68%) with fish oil</td>
<td>NE</td>
<td>↓ (~8%) Factor X with fish diet</td>
<td></td>
</tr>
<tr>
<td>Lorenz et al [291]</td>
<td>8 HS 1:0</td>
<td>Cod liver oil (20mL)/d (4g EPA/ 6g DHA) vs. Control diet</td>
<td>25 days</td>
<td>↓collagen-induced ↓ADP-induced</td>
<td>↓ (~21%) TX</td>
<td>↑ (~28%) Bleeding time ↓ (~11%) Platelet count</td>
<td></td>
</tr>
<tr>
<td>Terona et al [290]</td>
<td>8 HS 1:0</td>
<td>Purified EPA (75% EE)</td>
<td>4 weeks</td>
<td>↓collagen-induced</td>
<td>NE</td>
<td>↓ Blood viscosity</td>
<td></td>
</tr>
<tr>
<td>Von Schacky et al. [292]</td>
<td>6 HS 1:0</td>
<td>EPA-rich cod liver oil (10-40ml/day)</td>
<td>5 months</td>
<td>↓collagen-induced</td>
<td>↓ in subjects with high baseline levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Von Schacky et al. [294]</td>
<td>7 HS 1:0</td>
<td>Cross-over EPA-oil (6g/d) vs. DHA-oil (6g/d)</td>
<td>6 days</td>
<td>↓collagen-induced with EPA-oil ↓collagen-induced with DHA-oil ↓ADP-induced with DHA-oil</td>
<td>NS</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Protocol</td>
<td>Dose</td>
<td>Duration</td>
<td>Outcome 1</td>
<td>Outcome 2</td>
<td>Outcome 3</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>----------</td>
<td>------</td>
<td>----------</td>
<td>-----------------------------------</td>
<td>-----------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Galloway et al.</td>
<td>6 HS</td>
<td>1:0</td>
<td>EPA 1.8g/d</td>
<td>4 weeks</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Conquer et al.</td>
<td>12 HS</td>
<td>1:1</td>
<td>DHA oil (1.6g/d) vs. Placebo (corn oil)</td>
<td>6 weeks</td>
<td>NS</td>
<td>NS (TX)</td>
<td>NS (serum viscosity, Fb, factor VII)</td>
</tr>
<tr>
<td>Guillot et al.</td>
<td>12 HS</td>
<td>1:0</td>
<td>DHA 0.2g/d vs. DHA 0.4g/d vs. DHA 0.8g/d vs. DHA 1.6g/d</td>
<td>2 weeks</td>
<td>↓collagen-induced with 0.4g/d and 0.8g/day DHA</td>
<td>↓ TX with 0.4g/d and 0.8g/day DHA</td>
<td>NS with 0.2g/d or 1.6g/d</td>
</tr>
<tr>
<td>Harris et al.</td>
<td>8 HS</td>
<td>1:0</td>
<td>Fish oil 4.5g/d (2.7g EPA/1.8g DHA) vs. Aspirin (485mg) vs. Fish oil + aspirin</td>
<td>2 weeks</td>
<td>↓collagen-induced with fish oil</td>
<td>↓collagen-induced with aspirin</td>
<td>NE</td>
</tr>
<tr>
<td>Woodman et al.</td>
<td>51 HT, T2DM subjects</td>
<td>13:4</td>
<td>EPA 4g/d (96% EE) vs. DHA 4g/d (92% EE) vs. Placebo (olive oil)</td>
<td>2 weeks</td>
<td>↓(16.9%) collagen-induced with DHA vs. placebo</td>
<td>↓(-18.8%) TX with DHA vs. placebo</td>
<td>NS with EPA</td>
</tr>
<tr>
<td>Hostmark et al.</td>
<td>64 HS</td>
<td>1:0</td>
<td>Fish oil 14g/d (26% EPA/21% DHA) vs. Placebo (olive oil)</td>
<td>6 weeks</td>
<td>NE</td>
<td>NE</td>
<td>↓Fb (13.2%)</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Ratio</td>
<td>Intervention</td>
<td>Duration</td>
<td>Placebo</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
<td>-------</td>
<td>-------------------------------------</td>
<td>----------</td>
<td>---------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Vanschoonbeek et al. [359]</td>
<td>25 HS</td>
<td>1:0</td>
<td>Fish oil 3g/d (35% EPA /25% DHA)</td>
<td>4 weeks</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Eschen et al. [334]</td>
<td>60 HS</td>
<td>7:5</td>
<td>Fish oil 6.6g/d (3.0g EPA /2.9g DHA) vs. Fish oil 2.0g/d (0.9g EPA /0.8g DHA) vs. Placebo (olive oil)</td>
<td>12 weeks</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Nomura et al. [360]</td>
<td>20 HS vs. 41 HL,T2DM patients</td>
<td>33:28</td>
<td>EPA 1.8g/d</td>
<td>4 weeks</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Hansen et al. [361]</td>
<td>224 HS</td>
<td>1:0</td>
<td>EPA 3.8g/d (95% EE) vs. DHA 3.6g/d (90% EE) vs. Placebo (corn oil)</td>
<td>7 weeks</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Treatment</td>
<td>Duration</td>
<td>Intervention</td>
<td>Outcome</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>----------</td>
<td>-----------</td>
<td>----------</td>
<td>--------------</td>
<td>---------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Finnegan et al. [362]</td>
<td>150 HL subjects</td>
<td>3:2</td>
<td>EPA + DHA (0.5g) enriched spread vs. EPA + DHA (0.5g) spread + fish oil capsules (0.8g) vs. n-6PUFA spread (sunflower + safflower oil)</td>
<td>6 months</td>
<td>NE</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Oosthuizen et al. [363]</td>
<td>20 HS</td>
<td>1:1</td>
<td>Fish oil 6g/d vs. Placebo (olive oil)</td>
<td>6 weeks</td>
<td>NE</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Marckmann et al. [285]</td>
<td>50 HS</td>
<td>1:0</td>
<td>EPA+DHA (0.9g) spread vs. n-6PUFA spread (sunflower oil)</td>
<td>4 weeks</td>
<td>NE</td>
<td>NE</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ADP, adenosine diphosphate; DHA, docosahexaenoic acid; EE, ethyl ester; EPA, eicosapentaenoic acid; ETP, endogenous thrombin potential; Fb, fibrinogen; HL, hyperlipidemic; HS, healthy subjects; HT, hypertensive; ICAM, intracellular adhesion molecule; NE, not evaluated; NS, not significant; PAI-1, plasminogen activator inhibitor-1; PAF, platelet activating factor; P-sel, P-selectin; T2DM, type 2 diabetes mellitus; tPA, tissue plasminogen activator; TX, thromboxane; vWF, von Willebrand Factor.
1.3.4.1. *Eicosanoid modification, thromboxane and bleeding time*

The anti-thrombotic properties of FOs are commonly attributed to EPA as it is preferentially incorporated into platelets [205] compared with DHA which is an important factor to compete with AA metabolism in the COX and LOX pathways. Accordingly, both EPA and DHA suppress AA-induced TXA$_2$ production by platelets, while only EPA generates TXA$_3$, suggesting that EPA, but not DHA is a direct substrate for the COX/TX synthase [357]. Whilst DHA is not a substrate for COX, studies suggest that purified DHA also reduces platelet aggregation [275, 294, 357, 364]. This suggests that DHA may also exert direct effects on platelet function that is independent of eicosanoid production. Of the earlier studies, Siess et al [221] reported the TX lowering effects paralleled with the reduction in platelet aggregation in seven healthy Caucasian male subjects following a one week mackerel diet. Marked changes were observed in the ratio of EPA: AA relative to baseline values where EPA in plasma and platelet membranes was significantly increased coupled with a drastic reduction of AA. The investigators suggested that the diminished conversion of AA to TXA$_2$ by competitive inhibition of the platelet COX by EPA could be responsible for the platelet inhibitory response. Essentially, the study also demonstrated that it was possible to induce in western Europeans the ‘eskimotype’ pattern of platelet membrane FA through mimicking the diet of the eskimo by substituting mackerel as the sole source of their dietary fat. Lorenz and colleagues [291] later demonstrated that an unchanged western diet (typically high in AA) supplemented with LCn-3PUFA could induce the same favourable eicosanoid shift towards less reactive platelets. Lorenz et al [291] reported similar findings of a rapid incorporation of LCn-3PUFA into plasma and platelet PL following 25 days of cod liver oil supplementation. Eight healthy male subjects ingested 20mL cod liver oil daily equivalent to 4g EPA and 6g DHA in conjunction with their normal western diet. At post-intervention, concomitantly with the reduction of AA in platelet membranes, a reduction in TXA$_2$ and platelet aggregation was observed as well as a prolonged bleeding time and decreased platelet count. Though findings from these studies demonstrated LCn-3PUFA to be effective in TXA$_2$ reduction, the independent effects of EPA and DHA cannot be clarified.

Shortly after, Bruckner and co-workers [365] attempted to elucidate the effects of EPA versus DHA on prostaglandin biosynthesis and related thrombotic parameters in
rirs. Interestingly, the investigators showed that DHA-rich shark liver oil was less
effective than EPA-rich oil in suppressing TXA₂ synthesis and platelet aggregation;
however relative to AA, bleeding time or platelet aggregation was not significantly
different. While LCn-3PUFA has shown to alter hematologic parameters in humans,
this study suggested that the rat is not similarly affected. The authors concluded that
EPA and not DHA is responsible for the modifications in platelet prostaglandin
biosynthesis in the rat; however, these observations may not be directly applicable to
other species. Croft et al [366] further clarified the effects of EPA and DHA on
prostaglandin biosynthesis in rats by showing that the EPA-enriched diet was more
effective in decreasing AA in plasma and tissue PL content whereas rats fed the
DHA-enriched diet though showed increased serum DHA, but little effect on renal
PL FA. In addition, TX and vascular prostacyclin synthesis was diminished by 65%
and 36%, respectively in the EPA diet and no effects were reported in the rats fed
DHA-enriched diets. Thus it was concluded that in the rat, DHA exerts minimal
effect on prostaglandin synthesis when given as a dietary supplement.
In human subjects, Terona and colleagues [290] also reported similar effects where
dietary EPA was inefficiently metabolised to DHA. Terona et al reported that
administration of purified EPA (75% ethyl ester) for 4 weeks in male subjects
markedly increased EPA in platelet PL however did not change DHA or AA though
platelet aggregation was reduced. However, a series of studies conducted by von
Shacky and colleagues [292] reported contrasting findings in human subjects when
examining dietary LCn-3PUFA on eicosanoid formation. Six healthy males who
supplemented their diet with 10-40mL/day EPA-rich cod liver oil for 5 months had
marked increases in plasma DHA. Both EPA and DHA content increased rapidly in a
dose-related manner at the expense of AA in plasma PL and platelet PL. Collagen-
stimulated platelet aggregation and platelet counts also decreased significantly in
parallel to the changes in PL. However TX levels were unaltered in subjects with
normal TX baseline control levels. The investigators acknowledged the use of fish
oils containing both EPA and DHA and thus the inability to draw comparative effects
of the platelet responses observed. Consequently, in an attempt to discriminate
between the anti-aggregatory effects, a short term randomized cross-over study [294]
was conducted which compared the metabolism and effects on platelet function with
6g/day of EPA versus DHA in seven healthy males. The retroconversion
phenomenon of DHA to EPA was reported for the first time. Supplementation with
DHA increased EPA plasma PL. Conversely, EPA supplementation resulted in increased EPA plasma and platelet PL content where DHA levels were not altered. Interestingly, DHA supplementation resulted in a greater reduction in platelet aggregation relative to EPA. Importantly, EPA levels in the platelets remained constant during DHA supplementation therefore the anti-aggregatory effects observed cannot be attributed to EPA resulting from retroconversion. Though TX levels were unaltered in both LCn-3PUFAs, these findings demonstrated that inhibition of platelet aggregation is attributable in part to DHA.

Galloway et al [356] reported selectivity in the incorporation of EPA into membrane PL in 6 male subjects. EPA content of PC and PE increased following 1.8g/day of EPA for 4 weeks. However no incorporation of EPA into PI or PS was observed. Consistent with this, AA was reduced in PC and PE but remained unchanged in PI and PS of the membrane. No significant changes in platelet aggregation responses or in TX levels were observed. The authors suggested that the incorporation of EPA into the platelet membrane PL is selective for PI thus the reported effects of EPA on platelet prostaglandin biosynthesis cannot be explained on the basis of its incorporation into and mobilization from the platelet membrane PL pool.

With smaller doses of LCn-3PUFA, contradictory results have been reported. Considerably small amounts of EPA have been observed to decrease aggregability and TX without changes in FA distribution in platelets [272, 367]. In contrast, other studies have reported significant increases in FA composition without changes in platelet aggregation or AA [368]. In addition, prolonged bleeding times and decreased platelet aggregation have been observed in males subjects several weeks after cessation of the fish diet where the FA composition have returned to pre-diet levels [295, 297]. The authors reported that their observations suggest that such fish diets do not delay hemostasis by suppressing formation of TXA2 locally nor directly by decreasing platelet aggregation or directly by the induced changes in LCn-3PUFA content [297].

Mori et al [275] compared the effects of dietary fish and fish oil capsules on platelet aggregation and TXA2 formation in 120 male subjects for 12 weeks. The investigators reported that collagen induced platelet aggregation and TXA2 were similarly reduced by LCn-3PUFA whether ingested as fish meals or fish oil capsules.
At 12 weeks post intervention, a 5-7% reduction in platelet aggregation and 6-11% decrease in platelet TXA<sub>2</sub> was reported. Furthermore, TXA<sub>2</sub> release from aggregated platelets was inversely correlated with total dietary LCn-3PUFA levels. The authors concluded that EPA may alter TX metabolism and DHA may act directly at the membrane level in a mechanism related to PL modification. However, still it cannot be drawn from this study, whether the anti-aggregatory effects in conjunction with TX lowering are more attributable to EPA or DHA. Though the dietary fish meal was calculated to provide approximately 1.32g EPA, the FA composition of the fish was variable and in particular for DHA which varied from 1.3 to 2.4 g/day. The ratio of EPA to DHA also shifted considerably within the fish diet groups, and consequently as a whole differed to that of the fish oil group. However the study was not designed to determine whether EPA and DHA differ in their effect on platelet function although the authors did acknowledge there is evidence suggestive of biological differences between the two fatty acids.

Various approaches have been undertaken to study the effect of DHA on platelet activity. *In vitro* and animal studies that have been carried out to evaluate the effect of incubations with exogenous DHA have been more promising. Rao and co-workers in 1983 [369] were among the first to report inhibition of platelet aggregation by exogenous DHA. In platelet rich plasma (PRP), DHA at 150 µM concentration inhibited platelet aggregation induced by AA at 450 µM. At the same concentration, DHA also inhibited the second wave of aggregation induced by adenosine diphosphate (ADP) and thrombin. This study demonstrated that DHA is able to induce a platelet inhibitory response against a 3-fold greater concentration of AA and concluded that DHA inhibited the conversion to TXA<sub>2</sub>. Srivastava [370] observed a dose-dependent inhibition of platelet aggregation by DHA in human platelets at concentrations of 12.5 – 500 µM and reduced formation of TXA<sub>2</sub> at all concentrations. McLennan et al demonstrated that DHA was more effective than EPA at inhibition of TX-induced contraction in the aortas of hypertensive rats [371]. In addition, studies have shown DHA to be more effective than EPA at inhibiting COX [372] and modifying platelet responsiveness to membrane TXA<sub>2</sub> receptor agonist [373]. Enrichment of platelet membranes with DHA *in vitro* resulted in a considerable decrease of platelet TXA<sub>2</sub>/Prostaglandin H2 receptor binding affinity whereas EPA caused only a minor effect [374]. Furthermore, Croset and Lagarde
[375] reported that the dosage of collagen and thrombin required to achieve 50% aggregation was significantly higher with DHA treated platelets. Human platelets enriched with DHA exhibited greater inhibition of platelet aggregation than either EPA alone or a combination of EPA and DHA. The combination of EPA and DHA led to less inhibition of platelet aggregation relative to DHA alone.

In agreement with animal [376, 377] and in vitro studies, a number of human studies suggest that DHA is a more potent anti-aggregatory agent than EPA at high doses [202, 294, 378]. Agren et al [355] compared the effects of a fish-enriched diet with either fish oil or a DHA-rich oil on platelet aggregation and hemostatic factors. No changes in platelet aggregation or hemostatic factors were observed in the male subjects consuming 1.68 g/day DHA–rich oil after 15 weeks. The authors concluded that moderate amounts of LCn-3PUFA in the form of fish diet or fish oil inhibit platelet aggregation however without effects on hemostatic factors and furthermore EPA-free DHA-oil is not effective for inhibition of platelet aggregation.

Conquer and Holub [208] also showed that a similar dose of 1.62g/day of DHA oil devoid of EPA for 6 weeks failed to reduce platelet aggregation in male and female subjects. Despite increases in DHA in PL and EPA due to metabolic retroconversion and at the expense of AA; collagen-induced platelet aggregation, TXA2 formation, fibrinogen and factor VII levels were not significantly altered by DHA supplementation. At higher doses, 6g/day DHA over 6 days reduced platelet aggregation to both collagen and ADP in healthy male subjects [294], however with unaltered TXA2 levels. In contrast, at the same dose, Ferretti et al [379] reported that DHA given as 6 g daily to healthy male subjects for 90 days produced a reduction in urinary excretion of TXA2 without changes in platelet aggregation or coagulation [282]. However, more recently Guillot et al reported that, 8 weeks supplementation with DHA alone decreased platelet aggregation in healthy males for doses as low as 0.4g/day whereas a higher dose of 1.6 mg/day was not effective [357].

Several studies have implied that inhibition of TX production is not the sole mechanism whereby LCn-3PUFAs exert their effects. Several studies that have been undertaken to examine the effects of LCn-3PUFA and aspirin have reported an additive effect on platelet aggregation, implying that LCn-3PUFA and aspirin affect hemostasis via different mechanisms. Given that the exclusive mode of action in
aspirin is the complete blockage of TXA$_2$ synthesis, this implies LCn-3PUFA possess several roles in influencing platelet response. Thorngren et al [296] showed in a study of 10 healthy male subjects, an additive effect on the platelet inhibitory response with administration of aspirin following a 11 week fish diet containing 2-3 g/day of EPA. In a subsequent report, the additive effect was shown for the prolongation of the bleeding time which was prolonged to a greater extent by a combination of aspirin and LCn-3PUFA than by either alone [295]. However the increase of EPA and DHA and decrease of AA in the platelet PL FA composition and the prolonged bleeding times did not parallel the reduction in platelet aggregation. Harris et al [358] confirmed these same effects in a short term 2 week study in 8 male subjects supplemented with capsules containing 2.7 g EPA and 1.8 g DHA. In addition, the authors also reported that aspirin versus LCn-3PUFA inhibited \textit{ex vivo} platelet aggregation to the same extent.

### 1.3.4.2. Procoagulant activity and coagulation factors

The beneficial effect of LCn-3PUFA on thrombotic disease risk is multifactorial and may partly rely on their anti-coagulant action. The Atherosclerosis Risk in Communities (ARIC) Study, comprised of 14,571 men and women, showed negative associations of EPA, DHA and DPA with plasma levels of fibrinogen, factor VIII, and vWF [380]. However while some studies point to a moderate reduction by LCn-3PUFA of plasma levels of fibrinogen and factors V, VII and X [363, 380, 381], other studies fail to detect this [128, 166]. The Northwick Park Heart Study utilising a cohort of 1511 males concluded that plasma fibrinogen levels may not be very responsive to short term dietary changes [251].

Hostmark and colleagues [215] were the first to report the reduction of plasma fibrinogen concentrations by dietary FO supplementation for six weeks. The investigators conducted a double blinded placebo controlled trial with 14g/day FO (25.7g EPA/20.5g DHA) against 14g olive oil in 64 male subjects. In the FO group, a significant decrease in fibrinogen concentrations (13.2%) was observed already at three weeks and maintained without further changes at six weeks. Plasma fibrinogen levels returned to baseline values at three weeks post-intervention; no changes in fibrinogen concentrations were observed in the olive oil placebo group. Shortly after, Raddack and colleagues also reported marked reductions in fibrinogen levels
with FO after eight weeks and further demonstrated that the reduction was dose-dependent. Though the sex of study subjects were not reported, interestingly, the reductions were greater in subjects with higher baseline fibrinogen values [382]. Other studies have also reported a similar finding where fibrinogen and FX levels were shown to be lowered with fish oil in those with higher respective baseline levels and hypercoagulant profiles [363, 380, 381]. Indeed variable hypocoagulant effects of fish oil supplementation have been previously described by Vanschoonbeek et al where the lowering effects of fish oil on fibrinogen were clustered around subjects with high fibrinogen carrying a structural fibrinogen α-chain polymorphism [359]. Thus a possible explanation for the lack of effect in fibrinogen or FX reported in studies may be that baseline concentrations were at the lower end of normal, and subjects with higher concentrations may be necessary to demonstrate a decline. Vanschoonbeek and colleagues also investigated the influence of fish oil on thrombin generation (reflecting the coagulant potential) and the factors involved. The study was conducted in twenty-five male subjects that received 3g of n-3PUFA (35% EPA: 25% DHA) daily for four weeks. In some, but not all subjects, fish oil supplementation decreased thrombin generation where the levels of fibrinogen and FV contributed to the variation [359]. Thus the degree of the decreased procoagulant effect of LCn-3PUFA supplementation may depend on fibrinogen levels. Factors VII and X did not influence thrombin generation and furthermore were not affected by LCn-3PUFA supplementation. The finding from this study contradicts others which proposed that LC n-3PUFAs may interfere with vitamin K action where factors VII and X were moderately reduced [285, 363, 380].

Thrombin generation strongly stimulates platelet procoagulant response [383], acting in concert with vWF [384]. Endothelium derived vWF and platelet derived P-selectin are integral in the initial stages of platelet adhesion to the endothelium for activation and even in the absence of thrombin, can trigger the procoagulant response [79, 385]. The reduction of cellular adhesion molecules (CAM) by LCn-3PUFA to limit platelet activation has also been reported. It has been demonstrated that the ability of PUFA to reduce levels of CAM increased with the degree of unsaturation, and that DHA was the most potent inhibitor of endothelial activation [386]. In addition, Eschen et al [334] reported that DHA content of granulocyte is negatively associated with ICAM-1 levels, while granulocyte content of EPA is not correlated with these ICAM-1 or
VCAM-1 levels. The decrease of P-sel was more marked in men when supplemented with 6.6g/d of n-3PUFA (3.0 g EPA: 2.9g DHA) for 12 weeks. Interestingly, the authors speculate that the effects of LCn-3PUFA on CAM may be dependent on gender and LCn-3PUFA dose [334]. Woodman and colleagues [202] in 2003 were the first to conduct a controlled study comparing the effects of purified EPA and DHA on platelet aggregation, activation and fibrinolytic function. Collagen-induced platelet aggregation and TXA₂ were reduced with DHA but not EPA supplementation however without effects on PAI-1, P-sel or vWF. However the study was in hypertensive, type 2 diabetic patients where platelet aggregation is affected differentially [387]. In contrast Park et al reported that mean platelet volume (a marker of platelet activation) and platelet count was decreased by 4g/day EPA and not DHA supplementation in healthy subjects over four weeks [281]. Similarly, Saga et al observed reduction of platelet count and platelet size with highly purified EPA in male subjects [388].

In the process of platelet activation, microparticles are shed from the remnant platelets resembling similar procoagulant PL surfaces. Though the effects of LCn-3PUFA supplementation on platelet MP in healthy individuals are lacking, 4.3g/day of combined EPA/DHA supplementation in patients with previous myocardial infarction has been shown to significantly reduce both the number and procoagulant activity of platelet derived MPs over a 12-week period [389]. Nomura et al also reported reductions in platelet-derived MP levels and P-selectin in hyperlipidemic type 2 diabetic patients when administered with 1.8g EPA a day for 4 weeks [360], suggesting that EPA is effective in subjects with elevated MP and CAM levels.

### 1.3.4.3. PAI-1 and fibrinolysis

Few studies have been conducted to investigate whether LCn-3PUFAs many enhance the fibrinolytic system. Particular attention has been paid to plasminogen activator inhibitor type 1 (PAI-1) activity as an inhibitor of fibrinolysis. High PAI-1 levels are associated with increased risk of arterial disease either causally or as a marker [390]. Similarly, there is no consensus on the effects of LCn-3PUFA on fibrinolysis. While some studies report that LCn-3PUFAs reduce the activity of PAI-
1 to indicate an improvement in fibrinolysis [391-393], others report no changes [394-398] or even significant increases in this variable [313, 363, 399-404].

Woodman et al comparing the independent effects of EPA and DHA in hypertensive, diabetic subjects reported no significant effects on plasma PAI-1, tPA or the tPA/PAI-1 ratio [202]. Toft and colleagues also observed that in hypertensive patients, supplementation 4g n-3PUFA/day for 16 weeks did not specifically increase PAI-1 or tPA activity compared with similar intake of n-6PUFAs. However, an increase of fibrinogen levels was observed in both groups [405]. Similarly, Hansen et al did not observe changes in PAI-1 activity with either 3.8g/day EPA or 3.6g/day DHA for seven weeks in 224 healthy male subjects. Plasma PAI-1 activity was not correlated with dietary intake or serum concentrations of n-3PUFAs [361].

Mehta et al reported that n-3PUFA intake for 4 weeks caused a fall a PAI-1 activity that paralleled a triglyceride lowering effect in CAD patients and normal subjects [392]. However, Schmidt and colleagues suggested that fish oil may inhibit fibrinolysis in normal subjects and patients with angina pectoris [395, 406]. A dose-dependent response of n-3PUFAs was observed in healthy male subjects where plasma fibrinogen levels decreased; however PAI-1 levels increased with higher doses of up to 9g/day n-3PUFA for 6 weeks [406]. In cardiac patients, supplementation of 4.5g/day n-3PUFA for 12 weeks also decreased fibrinolytic activity measured by the fibrin plate method [395]. Examining a cohort of 76 healthy male subjects, Emenis and colleagues stated that a moderate fish intake increases PAI-1 activity. The authors reported that a fish-enriched diet (135g mackerel paste containing 1.7g EPA: 3.0g DHA) for 6 weeks impaired fibrinolytic capacity by causing a 71% increase of the plasma PAI-1 levels and without changes in tPA [403]. The effects were not observed in the control group consuming a meat diet.

A long term 6 month supplementation study reported a lack of effect. Finnegan et al compared effects of dietary plant-derived ALA with EPA+DHA on fibrinolytic markers in moderately hyperlipidemic subjects. No differences in FVII, FXII, fibrinogen, platelet aggregation or PAI-1 levels were observed whether supplemented with an equivalent amount of 5.5g/day of ALA or EPA+DHA over 6 months [362]. However the absence of effect in this study may be owing to the use of
ALA as a control. Though shorter term studies have reported decreased fibrinolytic potential, interestingly, other long term studies have also reported no effects. Supporting the results of Finnegan et al, a study in 260 CAD patients also reported no changes in FVII, fibrinogen or PAI-1 activity with 4g/day LCn-3PUFA over 9 months [407]. Similarly, supplementation with 2g/day LCn-3PUFA for 5 months did not alter PAI-1 or tPA levels however a decrease in PAI-1 was observed in the corn oil control group [396]. A 4 month intervention in 25 healthy male subjects with 4g/day of LCn-3PUFA also reported no changes in fibrinogen, PAI antigen or PAI-1 levels [408].

Conversely, an extensive long term study over 7 years reported significant reduction of fibrinogen in 365 subjects supplemented with EPA-rich oils (MAX-EPA 18-19% EPA), however without changes in other hemostatic markers or platelet count [409]. The cohort was comprised of 304 males and 62 females with existing cardiovascular complications, thus this study established the safety of long-term LCn-3PUFA supplementation on the hematology in high risk patients. Interestingly, Oosthuizen et al reported that both fish oil (6g/day) and olive oil for 6 weeks lowered fibrinogen and FX while raising PAI-1 levels in female but not male subjects [363]. This study was a double-blinded cross-over study in healthy subjects of equal gender numbers; however female subjects had higher baseline fibrinogen, FX and PAI-1 levels compared to males. Additionally, this supports the study by Vanschoonbeek et al reporting fibrinogen lowering effects with higher respective baseline levels [359].

1.4. Gender-based nutraceutical approach: A possible Solution

While a plethora of studies supports the notion that LCn-3PUFA intake is a safe and efficacious means of reducing thrombotic disease risk, contrasting finding have diminished its potential. These disparate findings have been drawn from prospective and clinical studies which hold variation in LCn-3PUFA dose, concentration, intervention period as well as subject characteristics. The independent effects of EPA or DHA have not been explored. To date, there have been no controlled
supplementation trials which have directly compared only EPA versus only DHA at identical intakes. Furthermore, several studies have been conducted in cardiovascular disease patients, hypertensive or diabetic subjects where comparisons have been drawn despite their altered hemostatic balance. Few controlled studies have been conducted; however these studies ignored the role of gender as a confounding factor (Table 1-3).

As reviewed throughout this chapter, interplay of distinct sex differences is apparent from baseline hemostatic markers to platelet characteristics and from LCn-3PUFA plasma fatty acid metabolism to membrane phospholipid incorporation. Indeed sex hormones contribute substantially to the platelet response and ultimately cardiovascular outcome. Thus collectively these differences may influence the antithrombotic potential of LCn-3PUFA supplementation be it minimal, modest or marked. While gender-specific pharmacodynamic data are meager, evidence also supports the existence of sex-related differences. Supported by the wealth of evidence, it is speculated that males and females may respond differently according to supplementation with EPA or DHA. Thus a possible solution to redeem the antithrombotic effects of LCn-3PUFA as a safe and efficacious nutraceutical is a gender-based approach. Exploration into the independent effects of EPA and DHA in males and females will illuminate and provide insight into hitherto hidden mechanism. Ultimately this allows the identification of sex-specific outcomes and paves the way to optimal cardiovascular protection for both gender groups.

1.5. Research hypothesis and objectives

1.5.1. Original Hypothesis

It is not known whether the anti-aggregatory effects are owing to EPA or DHA and whether differences exist in the efficacy of individual LCn-3 PUFA to reduce platelet aggregation differentially. The majority of studies reporting conflicting results have used varying concentration ratios of EPA and DHA in their design. Thus my research hypothesis being that individual LCn-3PUFA (EPA, DPA, DHA) exert differential effects on platelet aggregation.
Objective 1: To determine and compare the effectiveness of EPA, DPA and DHA to inhibit platelet aggregation *in vitro* in healthy male and female subjects.

1.5.2. Preliminary novel findings

Objective 1 was achieved upon completion of my first study. Differential responses in the efficacy of individual Lcn-3PUFA to inhibit platelet aggregation were observed. However, a more striking and novel finding generated from my sample and data analyses were that not only there was there differential responses to each Lcn-3PUFA, but the responses were influenced in a gender-specific manner. Thus these findings from study 1 of my PhD research suggested that inhibition of platelet aggregation by Lcn-3PUFA is gender-specific. These novel results allowed my PhD research to significantly evolve and progress and became the rationale for my further studies. It was anticipated that only individual Lcn-3PUFAs exert differential effects on platelet aggregation, but gender is also a major determinant of response.

1.5.3. Updated hypothesis: a new perspective

In light of the view that males and females may benefit differently according to the type of omega-3 fatty acid supplementation, demonstration of gender-specific platelet responses may offer maximum cardiovascular risk reduction for both gender groups. Interestingly, the majority of the reported fish oil and platelet aggregation studies have ignored gender as a confounding factor and were predominantly conducted in males or in unequal gender groups. Not only are sex-related differences in CVD well established, but such sex differences in platelet parameters, platelet aggregation [317-319], anti-platelet response [410] and differences in lipid and Lcn-3PUFA metabolism [201] have been described. Disparate findings concerning effects of Lcn-3PUFA on platelet function may be explained by gender-specific differences in platelet aggregation in response to EPA or DHA. Irrespective of the bias caused by heterogeneity of the fish oil dosage, gender itself is an independent predictor of CVD outcome and thus represents a confounding factor to potential study bias. Thus the individual assessment of EPA versus DHA in addition with sex-specific analyses may likely explain the inconsistent results in the fish oil and platelet aggregation literature.
My research candidature examines and explores the differential mechanisms whereby the gender-based differences are expressed in response to omega-3 fatty acid supplementation. The broad hypothesis on which my candidacy is based is that gender-specific responses exist in platelet aggregation following LCn-3PUFA supplementation in healthy human subjects; more specifically males versus females are affected differentially following EPA or DHA supplementation.

1.5.4. Objectives:
This thesis puts forward an innovative perspective of the pathways underlying the cardio-protective effects of EPA and DHA. These series of research studies will allow insight for a better understanding of the mechanisms of gender-specific differences in platelet function for optimisation of treatment therapies. The primary objective of the study was to investigate the differential effects of LCn-3PUFA on platelet aggregation in an in vitro model and in an ex vivo clinical setting using a dual channel whole blood aggregometer in male and female human subjects. These studies will also likely explain the existing controversy in the scientific literature relating LCn-3PUFA and platelet aggregation.

The specific objectives of my candidacy were:

Objective 1: To determine and compare the effectiveness of EPA, DPA and DHA to inhibit platelet aggregation in vitro in healthy male and female subjects.

Objective 2: To compare the efficacy of acute dietary supplementation with EPA and DHA rich oils on ex vivo platelet aggregation in healthy human subjects and males vs. females.

Objective 3: To investigate the effects of acute supplementation with EPA or DHA rich oils on circulating platelet microparticle levels and procoagulant activity with respect to platelet aggregation and gender in healthy human subjects.

Objective 4: To examine the effects of long-term dietary supplementation with EPA or DHA rich oils on ex vivo platelet aggregation in healthy male versus female subjects.
Objective 5: To compare the effects of long-term dietary supplementation with EPA and DHA rich oils on plasma fatty acid profiles, procoagulant activity, coagulation factors and biochemical biomarkers of platelet aggregation in males vs. females.

1.5.5. Research significance and anticipated outcomes

It is hypothesized that the reported differences in platelet aggregation are due to hormonal and gender differences which has not been accounted for in previous studies. Hence, it is speculated that there will interactions between omega-3 fatty acids and sex hormones which will shed light on the mechanisms by which the gender based differences are expressed. Herein, a series of studies have been designed to elucidate the gender-biased hemostatic responses with EPA and DHA supplementation. These studies, for the first time have directly compared EPA versus DHA at identical intakes on platelet variables and stratified by gender.

If our hypothesis that males and females respond differently according to the type of omega-3 fatty acid supplementation is validated, it will assist in designing strategies to offer maximum cardiovascular risk reduction for both gender groups. Given the adverse effects associated with pharmacological agents, a safe and efficacious non-pharmacological approach is ideal inhibit platelet aggregation thereby reducing the risk of heart attacks, stroke and thrombosis. Therefore the outcomes generated from my research candidature may assist in designing a gender-based non-pharmacological strategy to reduce platelet and coagulant activity and ultimately reduce thrombotic risk and retard the atherosclerotic process. In addition, the outcomes of my research may also resolve a long standing controversy in the literature about the role of omega-3 fatty acids in thrombosis and related cardiovascular diseases. On an individual basis, a non-pharmacological approach will improve compliance and quality of life in addition to offering economic benefits by reducing the burden of disease.
Chapter Two – Methods


2.1. Research Study Design

Study 1 was an *in vitro* investigation designed to assess the effectiveness of LCn-3 PUFA (DHA, DPA and EPA) to inhibit platelet aggregation in healthy human subjects. Study 2 was designed as a blinded placebo controlled intervention trial where platelet function of healthy male and female subjects were monitored over a 24 hour period to assess the acute effects of supplementation with fish oil capsules on *ex vivo* platelet aggregation. The kinetics of the response to omega-3 fatty acids in males vs. females were studied as subjects donated blood samples at set time intervals over the 24 hour time period following supplementation. Study 3 was a double-blinded randomized placebo-controlled trial examining the effects of chronic supplementation with fish oils concentrates of low vs. high EPA to DHA ratios or placebo on *ex vivo* platelet function and coagulation biomarkers over a 4 week period.

2.2. Participants

Participants were recruited from the general community of Newcastle, Australia via advertisements on noticeboards of the University of Newcastle campus and through the Hunter Medical Research (HMRI) volunteer register. All participants provided written informed consent according to protocol guidelines approved by all applicable institutional and governmental regulations concerning the ethical use of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia, prior to commencing and registered with the Australia and New Zealand Clinical Trials Register. The studies were conducted in accordance with The Declaration of Helsinki.

2.2.1. Study 1

Participants that enrolled in the study were screened for eligibility prior to commencing. Eligible participants were healthy male and female adults 25 years of age or older. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological
disorders; body mass index (BMI) greater than 35 kg/m^2; taking aspirin or anti-
platelet medication or non-steroidal anti-inflammatory drugs. Participants were also
excluded if they had consumed fish oil supplements or consumed more than two
seafood servings/week within the previous 6 weeks. Participants were also asked to
complete a medical questionnaire and a 24 hour food recall prior to a blood sample
donation.

### 2.2.2. Study 2

Participants that were recruited for the study were healthy male and female adults 18
years of age or older that were able to participate and attend the research premises at
the Medical Sciences Building, The University of Newcastle, Australia, over a
consecutive 3 week period. Exclusion criteria were as per Study 1. Participants were
asked to complete a medical questionnaire, 24 hour food recall and follow a diet low
in tomatoes and seafood prior to the study day. All participants attended the research
unit on 3 separate occasions to consume a different supplement on each occasion
with a minimum one week washout period in between. Participants were blinded to
consume a single dose of 2 x 1g capsules containing either placebo (Sunola oil), or
EPA rich oil (EPAX 5510 TG/N) providing 1g EPA with an EPA/DHA ratio of 5:1,
or DHA rich oil (EPAX 1050 TG/N) providing 1g DHA with an EPA/DHA ratio of
1:5. During each visit, a fasting blood sample was collected prior to
supplementation, then 2, 5 and 24 hours following supplementation. The participants
remained in the research unit for the first 5 hours of the trial, and were then asked to
limit physical activity until the final 24 h sample was collected.

### 2.2.3. Study 3

Participants that were recruited for the study were healthy male and female adults 18
years of age or older that were able to participate and attend the research premises on
the start of their intervention and at the end of their four weeks. Exclusion criteria
were as per Studies 1 & 2. All participants attended the research unit at the Medical
Sciences Building, University of Newcastle, Australia on 2 occasions. On the first
visit participants completed a pre-trial medical history questionnaire and a 24 hour
food record prior to a fasting blood sample donation.
Participants were randomised using a computer-based random number generator with sex stratification to a treatment protocol of 2 x 1g capsules daily for 4 weeks containing either:

(i) Placebo (Sunola oil)

(ii) High EPA supplement [500:100mg EPA/DHA (EPAX 5510 TG/N)]

(iii) High DHA supplement [100:500mg EPA/DHA (EPAX 1050 TG/N)]

Participants and the co-investigators responsible for treatment allocation and assessment were blinded to the treatment groups. The supplements were masked and identifiable only by code to which only the principal study investigator had access to. Participants returned immediately after the intervention for a final fasting blood donation. Compliance to instructions and supplement intake was monitored via pre-intervention and post-intervention capsule count-back, telephone communication, analysis of plasma fatty acid composition and analysis of the daily record of capsule intake documented by participant.

2.3. Whole blood platelet aggregation

Venous blood was collected from the antecubital vein into 4.5mL vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson Biosciences Ltd, NSW AUS). All blood samples were analyzed 20 minutes after collection to allow stabilization. Platelet aggregation was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA, USA) according to the method of Cardinal and Flower, 1980 [411]. The principle of the whole blood aggregometry procedure uses impedance technology where an electrode probe assembly is inserted into cuvette containing a whole blood test sample. The electrode probe assembly consists of two metal wires that are immersed into the sample. An AC voltage in the millivolt range is applied to the probe circuit and the instrument measures the electrical resistance (impedance) between the two immersed wires. During a brief period of equilibration (6 min) at 37°C, a monolayer of platelets forms in the exposed portions of the wires resulting in a stable impedance value. An aggregating agonist is added to the cuvette and the stimulated platelets aggregate to the platelet monolayer in the immersed wires. The accumulation of platelets adds electrical resistance to the
circuit which is measured and quantified in ohms and the change in impedance is displayed as a function of time using the Chrono-Log AGGRO/LINK Software (version 5, Chronolog-Log, Halvertown PA, USA) (Figure 3-1). For all the aggregation assays, collagen (5µg/mL) was used as the agonist as platelet aggregation response is not affected with platelet abnormalities or secretion deficiencies [412, 413]. Furthermore, collagen is important in platelet aggregation studies as in vivo, damaged vascular endothelial surface exposes subendothelial collagen fibers initiating the aggregation response [105]. Thus it is considered the first aggregating or procoagulant factor that the platelet encounters following vascular trauma.

2.3.1. Study 1

Whole blood (498 µL) was diluted with an equivalent volume of phosphate-buffered saline to adjust for a final 1mL sample volume in a 1mL polystyrene cuvette (Chrono-Log Halvertown PA, USA). Ethanol (4 µL) was added to control samples for baseline aggregation comparisons with samples treated with 4uL DHA or DPA or DPA (Sigma- Aldrich, St. Louis, MO, USA) for a final 1.0µM concentration. A 1.0 µM concentration of n-3 PUFA was used for the study as it was determined to be most effective in the pilot study (data not shown). Controls and treated samples were pre-incubated for 6 mins at 37°C prior to stimulation with 5µg/mL collagen (DKSH Ltd, VIC, AUS). Platelet function was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. Blood samples collected from each participant were tested for all LCn-3 PUFA effects in platelet aggregation in vitro. To minimise bias in platelet reactivity due to cell viability, a control sample was run against each LCn-3 PUFA treated sample within a 15 min time frame. Inhibition of platelet aggregation was determined by calculating the change in AUC from LCn-3 PUFA treated samples with their corresponding controls and expressed as a percentage change.

2.3.2. Studies 2 and 3

Whole blood (500 µL) was diluted with an equal volume of phosphate-buffered saline in a 1mL polystyrene cuvette (Chrono-Log Halvertown PA, USA). Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5µg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation
curve (AUC) calculated. Reduction of platelet aggregation at 2, 5 and 24 hours post supplementation (study 2) and after 4 weeks (study 3) was determined by calculating the change in AUC from blood samples collected post supplementation with pre-supplementation baseline aggregation value and expressed as a percentage change.

**Figure 2-1. Example of platelet aggregation curve.** Platelet aggregation is measured in real time induced by addition of collagen and the area under the aggregation curve is calculated.

### 2.4. Manual platelet count and blood biochemistry

Venous blood was collected from the antecubital vein into 4mL vacutainer tubes containing K$_2$EDTA (Becton Dickinson Bioscience, Ltd, NSW AUS) for platelet count estimates and blood biochemistry. Measurement variation was minimized by having the same technician process all samples using the same equipment. Manual platelet count estimates were determined using differential Wright’s stained peripheral blood smears and phase contrast light microscopy techniques (World Precision Instruments Inc. Saratosa, FL) [414]. The thin blood smears were prepared
by placing 1-2 drops of blood (approx. 4mm) onto one end of the microscopic slide, the drop was spread by using another slide (spreader) and placing the spreader at a 45° angle and pushing the spreader across the slide to make the smear. The slide was allowed to air dry before fixed in methanol for 10 mins then straight Wright’s Stain for 3 mins and then in diluted Wright’s stain for 9 mins. The diluted Wright’s stain was prepared with 1:10 with Wright’s Buffer Concentrate (Thermo Fisher Scientific, NSW, AUS). Total cholesterol and human C-reactive protein (CRP) levels were measured in plasma and analyzed by the Hunter New England Area Health Pathology Services (NSW, AUS) using standard automated analytical techniques.

2.5. Full blood count and sex hormonal levels

Venous blood was collected from the antecubital vein into 4mL K2EDTA vacutainer tubes and lithium heparin tubes for analysis of full blood count and hormonal levels (testosterone, oestradiol), respectively. Samples were analysed by Hunter New England Area Health Pathology Services (NSW, Australia) using standard automated analytical techniques.

2.6. Measurement of TXB2, vWF, MP activity, P-sel and PAI-1

For measurement of TXB2, P-sel, PAI-1 and plasma fatty acid composition, EDTA blood was centrifuged at 3000 x g for 10 min (Heraeus Biofuge Stratos, Radiometer Pacific, AUS) to obtain platelet free plasma and aliquots were stored at -80° C until analysis. For measurement of MP and vWF activity, sodium citrate blood was centrifuged and stored as above. All plasma biomarkers were measured using commercially available immunoassay kits following the manufacturer’s instructions. Thromboxane B2 was quantitated using a competitive enzyme immunoassay (EIA) (Sapphire Bioscience, Waterloo, NSW, Australia), von Willebrand factor (vWF) activity was determined using a collagen binding assay (CBA) (Life Therapeutics, Clarkston, GA, USA), while MP activity (HYPHEN BioMed, Neuville-sur-Oise, France), soluble P-selectin and PAI-1 levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (R &D Systems, Minneapolis, MN, USA). All
samples were analysed in duplicate and only those with a %CV < 15% were used in the final analysis. Replicate samples from individual participants were determined in the same series to avoid bias due to assay variability.

2.7. Determination of coagulation factor activity

For measurement of coagulation factors and thrombin generation, citrate blood was centrifuged at 3000 x g for 10 min to obtain platelet free plasma and aliquots were stored at -80° C until analysis. All samples were rapidly thawed in a water bath at 37 °C prior to analysis. Standard coagulometric methods were used to detect factors II, V, VII, VIII, IX and X levels (%) on a Behring Coagulation System analyser (BCS; Siemens Healthcare Diagnostics Pty, Ltd. VIC, AUS), and fibrinogen assays were performed on the Sysmex CA-1500 coagulation system analyser (Dade Behring Inc. Newark, DE) using kits and reagents purchased from Dade Behring. Briefly, each participant’s individual plasma sample was mixed with the respective coagulation factor deficient plasma and a recombinant thromboplastin (Innovin) or activated partial thromboplastin time (Actin FSL) reagent and the time for clot formation measured in seconds. The amount of factor activity in the system was then quantified from a standard curve of the corresponding coagulation factor deficient plasma and known concentrations of a normal plasma plotted against the clotting time. Results are expressed as levels or activities (% of norm).

Plasma von Willebrand factor (vWF) activity was determined using a collagen binding assay (CBA) (Life Therapeutics, Clarkston, GA, USA) following the manufacturer’s instructions. vWF antigen (vWF:Ag) was measured on a BCS according to standard immunoturbidmetric methods as per the manufacturer’s instructions. Briefly, a suspension of polystyrene particles coated with vWF-specific antibodies was mixed with each participant’s plasma, and agglutination was detected via increase in turbidity which was proportional to the antigen level present in the participant’s sample.
2.8. **Endogenous Thrombin Potential (ETP)**

Thrombin generation was performed at 37°C on a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Finland) and analysed using the Calibrated Automated Thrombogram (Thrombinoscope BV, Maastricht, the Netherlands) according to the manufacturer’s instructions. An aliquot of 80 µl of platelet-free plasma was diluted with 20 µl of PPP reagent (containing 5 pM recombinant tissue factor and 4 µM phospholipids), and 20 µl of FluCa solution (Hepes, pH 7.35, 100 nM CaCl₂, 60 mg/mL bovine albumin, and 2.5 mM Z-Gly-Gly-Arg-amido methyl coumarin). The mean AUC corresponding to the ETP (nM x min) of duplicate tests was used for analysis. The intra-assay variability was calculated to be 1.7%.

2.9. **Flow cytometry**

Platelet MP was identified and quantified by flow cytometry with specific platelet (CD41a) surface markers. All reagents and solutions used were sterile and filtered (0.2-µm filter). A 30-µl aliquot of platelet-free plasma was incubated at room temperature for 15 min with 10 µl of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA) and 10 µl of Annexin V–FITC (BD PharMingen, San Diego, CA, USA) as a marker of activation/PS expression. The sample was incubated for 30 min at room temperature and then diluted with 500 µl of PBS and stained with 30-µl fluorospheres (Flow-Count, Beckman Coulter Inc., CA, USA). A known number of 10-µm enumeration beads (Flowcount Fluorospheres; Beckman Coulter, Fullerton, CA, USA) were added prior to analysis, and data were analyzed using a BD FACS Canto flow cytometer and CXP software (BD Biosciences, CA, USA) according to a standardized method [415]. The MP gate, based on a particle size of 1.0 µm, was used for identification of MP. Events in the MP gate were assessed for labelling with antibody-positive events to distinguish true events from background noise. The number of CD41+ Annexin V bound MP was identified as MP expressing procoagulant activity. Microparticles were quantified using a modified Combes method [416] and enumerated by using the formula:

\[
\text{MP count} = \frac{\text{bead count} \times \text{bead concentration}/\mu L}{\mu L}
\]
2.10. Plasma fatty acid analyses

For measurement of plasma fatty acid composition, EDTA blood was centrifuged at 3000 x g for 10 min and aliquots were stored at -80°C analysis. The fatty acid composition of plasma lipids was determined according to a modification in the method of Lepage & Roy [417] using an acetyl chloride methylation procedure. A 2mL volume of methanol: toluene (4:1) containing 4ug/mL C19:0 internal standard (Nu Check Prep, Elysian, MN, USA) was added to a 100 µL aliquot of plasma. Acetyl chloride (200 µL) was added drop wise before heating at 100ºC for 60 min then rapidly cooled. The sample was allowed to stand for 5 min with addition of 5 mL 6% K₂CO₃ solution and vortexed. After centrifugation at 300 xg for 10 minutes the upper toluene supernatant phase containing the fatty acid methyl esters was collected. Fatty acid methyl esters were quantified using GC (Hewlett Packard 6890; Hewlett Packard, Palo Alto, CA, USA). The identity of each fatty acid peak was ascertained by comparison of the peaks’ retention time with those of synthetic standards of known fatty acid composition (Nu Check Prep, Elysian, MN, USA). The relative amount of each fatty acid was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids.

2.11. Anthropometry and Food Intake

The height and weight of each participant was measured at the commencement of the study. Body weight was determined using a calibrated balance beam scale (PCS Measurement, NSW, Australia) measured in 0.1kg increments, while participants wore light clothing and no shoes. Height was measured using a wall-mounted stadiometer, measured to the nearest millimeter. BMI was calculated as the weight in kilograms (kg) divided by the square of the height in meters (m) to the nearest 0.1 (kg/m²). Measurement variation was minimized by having the same technician to measure anthropometry using the same equipment. Participants provided an account of foods and beverages consumed over the last 24 hours prior to and following the dietary intervention to estimate dietary intake and foods that may influence blood
results. Dietary analysis was performed using nutrient analysis software (FoodWorks version 4.0, Xyris Software Pty Ltd, Australia).

2.12. Statistical analyses

Data calculations, sample size and statistical analyses were performed using SPSS software (version 16.0 and 19, SPSS Inc., Chicago) in studies 1 and 2 and IBM Statistics software (version 20, SPSS Inc., Chicago) in study 3. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test variables within gender and LCn-3 PUFA group. Variables that were not normally distributed were log-transformed prior to analysis. Statistical significance was assessed using one way ANOVA and the repeated measures t-test for paired data. Comparisons between gender groups and different LCn-3 PUFA groups were made with general linear models using two way univariate and multivariate ANOVA with post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated. Changes in hemostatic markers, platelet aggregation and plasma fatty acids were determined by calculating the difference from blood samples collected at post intervention with baseline values and expressed as a percentage change. The sample size for the comparison of platelet aggregation between males and females before and after treatment was performed using a power calculation. A two-tailed t-test and an alpha of 0.05 and a power of 0.98 were used in the calculation. The sample size was calculated to be 18 for study 1, therefore a total sample size of 40 (n = 40; 20 males and 20 females) in study 1 was required, including any dropouts. A sample size of 28 for studies 2 and 3 was calculated using means, standard deviation and power calculations from study 1 for comparison of platelet aggregation between males and females using a two-tailed t-test, an alpha of 0.05 and a power of 0.90. Based on this data the study was powered to detect a minimum 10 % change in platelet aggregation and allowed for a 10% dropout rate due for unforeseen circumstances. Therefore, a total sample size of 90 (n = 90; 30 per supplement group) in study 2 was required, including any dropouts.
Chapter Three - Investigation of differential effects of individual LCn-3PUFA on platelet aggregation *in vitro* in human subjects

The content of this chapter is covered by the publication entitled:


**Thesis objective addressed in this chapter:**

**Objective 1:** To determine the effectiveness of EPA and DHA to inhibit platelet aggregation *in vitro* in healthy human subjects and in males versus females.
3.1. Objective 1

3.1.1. Abstract
Existence of gender differences in CVD following LCn-3 PUFA supplementation have suggested that sex hormones play a role in cardio-protection. The objective of this study was to determine gender specific responses in the efficacy of LCn-3 PUFA to inhibit platelet aggregation in vitro. Blood was analyzed for collagen-induced platelet aggregation following pre-incubation with LCn-3 PUFA in healthy adults (n = 42). EPA was significantly more effective in reducing platelet aggregation compared with DPA and DHA. When grouped by gender, this differential pattern was followed in males only. In females, DHA, DPA and EPA were all equally effective. Between group analyses (LCn-3 PUFA vs. gender) showed that both DHA and DPA were significantly less effective in males compared with females. EPA was equally effective in reducing platelet aggregation in both groups. These findings show that significant gender differences exist in platelet aggregation in response to various LCn-3 PUFA treatments.

3.1.2. Introduction
Cardiovascular disease (CVD) is a leading cause of morbidity and mortality for both men and women worldwide; however, men are twice as likely to suffer from CVD than women [305]. Gender differences are apparent long before CVD appear, but the underlying mechanisms to explain these differences are not yet clear. The existence of gender differences in CVD has suggested that female sex hormones may play an important role in cardio-protection particularly in the child-bearing age range. Platelets play a pivotal role in the development of thrombosis and CVD and as a consequence, inhibitors of platelet aggregation are becoming increasingly important in the prevention and treatment of many atherothrombotic disorders [53]. It is well established that anti-platelet therapy such as aspirin and clopidogrel is of proven efficacy in the prevention of vascular events in high-risk patients [418]. However, despite such clinical benefits, currently available agents have some limitations. Resistance to anti-platelet medications are now emerging clinical entities with potentially severe consequences such as recurrent myocardial infarction and stroke [147]. There is also limited data addressing the long term safety and efficacy of anti-
platelet agents in high risk sub-groups such as diabetics [145]. Furthermore, potential drug-drug interactions have been described in retrospective clinical studies with higher cardiovascular death rates demonstrated in patients taking clopidogrel while on statin therapy [148]. As CVD continue to place a severe burden upon health, social, economic and emotional costs, there is an urgent need to develop safe and efficacious, non-pharmacological approaches to prevent clot formation and retard atherosclerotic process to reduce the risk of CVD.

Long chain omega-3 polyunsaturated fatty acid (LCn-3 PUFA) supplementation plays a significant role in minimizing cardiovascular events. Recent studies have demonstrated that dietary intake of LCn-3 PUFA can reduce coronary heart disease risk due to their anti-atherogenic, anti-inflammatory and anti-thrombotic effects [372]. Platelet aggregation is an early event in the development of thrombosis and is initiated by thromboxane A$_2$ (TXA$_2$), a potent aggregatory agent and vasoconstrictor [180]. Evidence from dietary intervention studies has found that consumption of LCn-3 PUFA decreases the production of TXA$_2$. [275, 283, 292, 372, 419, 420], prolongs bleeding time [295, 419] and decreases platelet aggregation in vitro [421]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce aggregation in hypertensive subjects [275], diabetic subjects [422, 423] and in healthy controls both in vitro and ex vivo [294, 424-427]. In the animal model, docosapentaenoic acid (DPA) suppressed TXA2 formation in a dose dependent manner [372].

Although an inverse association between LCn-3 PUFA and CVD risk is a consistent finding, a number of human clinical trials involving EPA and/or DHA supplementation have yielded unequivocal evidence for their effectiveness against platelet aggregation. Non modifiable determinants of CVD such as gender and age exist to influence LCn-3 PUFA metabolism that may explain the controversy in the literature about LCn-3 PUFA and platelet aggregation; an important determinant in atherosclerosis and thrombotic disorders. There is limited information directly comparing platelet variables with LCn-3 PUFA supplementation. Despite the several cardiovascular gender differences and outcomes [300, 301], most studies have been conducted in males only. The objective of the present study was to determine the efficacy of individual LCn-3 PUFA (EPA, DPA and DHA) to inhibit platelet aggregation in vitro and the extent to which LCn-3 PUFA may influence platelet
reactivity in healthy males and females. Previous in vitro aggregation studies have been carried involving exogenous addition of LCn-3 PUFA to platelets [293, 350, 370, 375, 424, 428, 429]. Exogenous addition of LCn-3 PUFA has been reported to be poorly oxygenated in vitro with efficient conversion to TXB3 (weaker aggregation agonist) [231, 293, 370] or the release of significantly less TXB2 [430] to effectively reduce platelet aggregation without incorporation into platelet phospholipids [430]. We studied in vitro platelet aggregation on the basis of these previous studies with EPA, DPA and DHA as these are all present in fish, fish oils and land animal sources of LCn-3 PUFA [431]. The recent availability of pure EPA, DHA and DPA has enabled further research potential to investigate the specific roles and health benefits of the individual LCn-3 PUFAs in platelet aggregation. We aim to produce findings that will allow scientifically sound recommendations to develop preventative and therapeutic strategies for different gender groups regarding cardiovascular health.

3.1.3. Study Design and Methods

3.1.3.1. Participants

This study is an in vitro investigation designed to assess the effectiveness of LCn-3 PUFA (DHA, DPA and EPA) to inhibit platelet aggregation in healthy human subjects as well as comparing the gender-specific responses to LCn-3 PUFA treatment. Eligible participants were healthy male and female adults 18 years of age or older. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m²; taking aspirin or anti-platelet medication or non-steroidal anti-inflammatory drugs. Participants were also excluded if they had consumed fish oil supplements or consumed more than two seafood servings/week within the previous 6 weeks. Participants were also asked to complete a medical questionnaire and a 24 hour food recall prior to a blood sample donation. All participants provided written informed consent according to protocol guidelines approved by all applicable institutional and governmental regulations concerning the ethical use of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia, prior to commencing (H-2008-0149). The study was conducted in accordance with The Declaration of Helsinki.
3.1.3.2. **Platelet function assays**

Venous blood was collected into vacutainer tubes containing 3.2% sodium citrate (for platelet aggregation assays) and EDTA tubes (for platelet count estimates and blood biochemistry). Measurement variation was minimized by having the same technician process all samples using the same equipment. All blood samples were analyzed 20 minutes after collection to allow stabilization. Manual platelet count estimates were determined using differential Wright’s stained peripheral blood smears and phase contrast light microscopy techniques (World Precision Instruments Inc. Saratosa, FL) [414]. Total cholesterol and C-reactive protein (CRP) levels were measured in plasma using standard analytical techniques. Plasma was obtained by centrifuging (Heraeus Biofuge Stratos, Radiometer Pacific, Australia) at 3000 xg for 10 min at 4°C. Platelet function (aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA) according to the method of Cardinal and Flower, 1980 [411]. Whole blood (498 µL) was diluted with an equivalent volume of phosphate-buffered saline to adjust for a final 1mL sample volume. Ethanol (4 µL) was added to control samples for baseline aggregation comparisons with samples treated with 4uL DHA or DPA or DPA (Sigma- Aldrich, St. Louis, MO) for a final 1.0 µM concentration. A 1.0 µM concentration of n-3 PUFA was used for the study as it was determined to be most effective in the pilot study (data not shown). Controls and treated samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5µg/mL). Platelet function was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. Blood samples collected from each participant were tested for all LCn-3 PUFA effects in platelet aggregation in vitro. To minimise bias in platelet reactivity due to cell viability, a control sample was run against each LCn-3 PUFA treated sample within a 15 min time frame. Inhibition of platelet aggregation was determined by calculating the change in AUC from LCn-3 PUFA treated samples with their corresponding controls and expressed as a percentage change.

3.1.3.1. **Statistical analysis**

Data calculations and statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago). All data are presented as mean ± SEM. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test
variables within gender and LCn-3 PUFA group. Variables that were not normally distributed were log-transformed prior to analysis. Statistical significance was assessed using one way ANOVA. Comparisons between gender groups and different LCn-3 PUFA groups were made with general linear models using univariate analysis and post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

3.1.4. Results
A total of 44 participants were initially recruited in the study; 2 male participants either did not comply with diet restrictions or received medication affecting blood coagulation. All study participants were recruited from the general community of Newcastle, NSW, Australia. Forty two participants completed the study; 20 males and 22 females. Males showed to have significantly greater weight (kg) and height (cm) measurements but were not significantly different when adjusted for BMI (kg/m²). Baseline aggregation values and CRP were also significantly different between male and female groups. Females showed to have significantly higher platelet count as well as significantly higher baseline aggregation than males by 33.9% and 20.8%, respectively (Table 3-1).
Table 3-1. Characteristics of the study participants

<table>
<thead>
<tr>
<th></th>
<th>Combined</th>
<th>Male</th>
<th>Female</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 42)</td>
<td>(n = 20)</td>
<td>(n = 22)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>34.6 ± 1.0</td>
<td>33.5 ± 2.1</td>
<td>35.7 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72.9 ± 2.3</td>
<td>80.1 ± 2.1</td>
<td>66.5 ± 3.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169 ± 13.3</td>
<td>176 ± 14.6</td>
<td>163 ± 11.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.2 ± 0.4</td>
<td>25.8 ± 0.6</td>
<td>24.7 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet count (x 10⁹/L)</td>
<td>220 ± 70.8</td>
<td>173 ± 11.8</td>
<td>262 ± 16.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Baseline aggregation (AUC)</td>
<td>44.9 ± 1.3</td>
<td>39.5 ± 2.2</td>
<td>49.9 ± 3.4</td>
<td>0.016</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.96 ± 0.1</td>
<td>5.08 ± 0.2</td>
<td>4.85 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.68 ± 0.3</td>
<td>2.65 ± 0.3</td>
<td>4.62 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; CRP, C-reactive protein; NS, not significant; *Values are mean ± SEM; †Independent Samples T-Test for males vs. female

Figure 3-1. Inhibitory effects of n-3 PUFA on platelet aggregation in males and females combined. Results are mean percentage inhibition using one-way ANOVA descriptives; values without a common symbol are significantly different, P < 0.001.
Preliminary investigations showed that all the LCn-3 PUFA inhibited platelet aggregation in comparison to oleic acid or ethanol (data not shown). Addition of ethanol (vehicle for fatty acid solubilization) alone or oleic acid had no significant effect on platelet aggregation. DHA and DPA (36.4% and 33.5% respectively) were equally effective in reducing platelet aggregation while EPA (51.7%) was the most effective with a significantly higher percentage inhibition ($P<0.01$ vs. DPA and $P = 0.004$ vs. DHA) (Figure 3-1). When the aggregation data was separated into male and female groups, significant gender specific responses were observed. It became apparent that the same pattern of inhibition of platelet aggregation by LCn-3 PUFA was followed only in the male group. Within group analysis showed that blood from males responded more effectively to EPA (48.9%) in comparison to DHA (25.3%) and DPA (21.7%) ($P=0.001$ and $P <0.001$ respectively). However in female participants, all the three LCn-3 PUFA inhibited platelet aggregation to a similar extent. DHA (46.5%), DPA (44.2%) and EPA (54.3%) were all equally effective in reducing platelet aggregation in females where no significant differences were observed. Between group analyses (LCn-3 PUFA vs. gender) showed that EPA was also equally effective in reducing platelet aggregation in the male vs. female group. Both DHA and DPA were significantly less effective in males compared with females ($P=0.002$ and $P < 0.001$ respectively) (Figure 3-2).

A strong positive correlation between baseline aggregation and platelet count was observed in males and females combined (Figure 3-3). When grouped by gender, males alone showed no correlation between baseline aggregation and platelet count, however a stronger positive correlation was observed in females (Figure 3-4). No correlation was observed between platelet count and percentage inhibition of aggregation by the LCn-3 PUFA.
Figure 3-2. Gender differences in response to LCn-3 PUFA in platelet aggregation. Results are mean percentage inhibition; values without a common symbol *, † are significantly different, \( P \leq 0.001 \).

Figure 3-3. Relationship between baseline aggregation and platelet count in males and females combined. Results are bivariate correlation and Pearson Coefficient for area under aggregation curve of control sample and manual platelet count estimates.
Figure 3-4. Gender differences in relationship between baseline aggregation and platelet count in males and females. Results are bivariate correlation and Pearson Coefficient for area under aggregation curve of control sample and manual platelet count estimates.

3.1.5. Discussion
In the present study, we have shown that significant gender differences exist in platelet function in response to LCn-3 PUFA treatment. To the best of our knowledge, this is the first study in humans to show gender-specific responses with individual LCn-3 PUFA to inhibit platelet aggregation. The results from our study show that all three of the LCn-3 PUFA are effective inhibitors of platelet aggregation in vitro. In the combined male and female population, EPA emerged as the most effective inhibitor which is consistent with published literature [426, 432-434]. DHA and DPA were equally, however significantly less effective in this role. The novel and most striking finding was that this differential pattern of LCn-3 PUFA effect was strictly followed in males only. In females, all three LCn-3 PUFA were extremely and equally effective, whereas EPA was the only effective LCn-3 PUFA in males. Since the anti-aggregatory effects of LCn-3 PUFA were shown to be more potent in females, it may be possible that hormonal differences influence factors or
mechanisms involved in the blood coagulation cascade. Therefore, our findings are suggestive that sex hormones may interact with LCn-3 PUFA in suppressing platelet activation pathways.

DHA and DPA treatment were as effective as EPA in female subjects only; clearly further work is required to investigate the mechanism of action to different responses to LCn-3 PUFA between male and female subjects. Confirmation of the lack of effect of DHA in male subjects comes from a study from Finland in male subjects [355]. These authors reported that a fish or a fish oil supplemented diet but not DHA inhibited platelet aggregation, leading the authors to conclude that DHA was not effective in decreasing platelet aggregation. Our results suggest that this conclusion is not applicable to the population as a whole but may be limited to male subjects. In another study, Park and Harris reported that EPA, but not DHA decreased mean platelet volume (MPV) in normal male and female subjects [281]. A decrease in MPV indicates a reduction in platelet activation; however gender was ignored as a confounding factor. In addition, a study by Nelson et al observed that dietary DHA produced no physiological changes in blood coagulation or platelet function in healthy male subjects [282]. The controversy relating LCn-3 PUFA supplementation and platelet aggregation may be resolved by re-examination of the existing literature in the light of our observations that the male subjects respond to EPA more effectively than DPA or DHA while females exhibit almost similar efficacy with all the three long LCn-3 PUFA.

It is well established that hormone-dependent gender differences exist in vascular function. Estrogen causes vasodilatation, affects blood pressure, inhibits the response of blood vessels to injury and retards the development of atherosclerosis [435]. In addition, lipid abnormalities contribute substantially to atherosclerosis and are also regulated by sex steroid hormones, principally by way of hepatic lipoprotein metabolism [340]. The ability of LCn-3 PUFA to modify and improve plasma lipid profiles offers vascular and cardio protection. However, the metabolic capacity of an individual to biosynthesize and mobilize EPA, DHA and DPA is important to enable the utilization of these fatty acids.
Gender differences in LCn-3 PUFA metabolism have also recently been reported. In a large population study, women had lower proportions of EPA and DPA in phospholipids and a higher proportion of DHA though the intake of fish fat did not differ between men and women [338]. In addition, evidence of gender differences in LCn-3 PUFA metabolism have been reported in studies using stable-isotope tracer techniques. Women, when fed $^{13}$C-labelled α-linolenic acid, had a greater capacity to synthesize $^{13}$C-labelled DHA compared to men [200]. Furthermore, whether fed a beef or fish based diets, the rates of conversion of ALA to DHA were significantly greater in women [348, 436]. This is consistent with our findings suggesting that females may be more responsive to alterations in long chain n-3 PUFA compared to males. Platelets of females are also reported to be intrinsically more sensitive than male platelets [320]. Indeed our results showed that females responded to all three LCn-3 PUFA to the same extent where males responded primarily to EPA. Furthermore, supporting our findings, a study by Cheryk et al demonstrated that the amount of EPA incorporated into platelet phospholipids was 3-4 fold higher than DHA and DPA in healthy males [350].

Mechanisms by which LCn-3 PUFA exert differential inhibitory effects in males versus females cannot be ascertained from the results presented in this study. At baseline, females had significantly higher platelet count and aggregation which are consistent with previous studies [317-319]. Baseline aggregation was positively correlated to platelet count in females but not in males. One might argue that greater baseline platelet reactivity in females allows for a more prominent reduction in platelet function when exposed to treatment with LCn-3 PUFA. However, the relatively greater reactivity of female platelets to the three LCn-3 PUFA was not caused by a lack of reactivity in male platelets since males responded equally to EPA. No other correlations were observed with platelet count suggesting that platelet count is not a determinant of response to LCn-3 PUFA treatment. Interestingly, plasma CRP levels were found to be higher in females compared to the male subjects, however no correlation between CRP level and platelet aggregation response with LCn-3 PUFA was observed. This eliminates the possibility of CRP involvement in the determination of platelet aggregation and subsequent response by LCn-3 PUFAs.
It is possible that platelet aggregation is dependent on platelet and/or aggregate size rather than platelet count. Larger platelets have a greater mass and are more reactive, produce more prothrombotic factors, and aggregate more easily when exposed to agonist [437]. Conventional aggregometry using a light scattering technique has shown that females have more medium aggregates while males have more small aggregates [438]. Furthermore, it has been shown that testosterone decreased only small aggregates while estradiol decreased all sizes of aggregates [410, 438]. Perhaps the inhibitory effect of LCn-3 PUFA may also act to reduce aggregate size in combination with sex hormones; or that larger platelets in females have a greater mass and therefore are metabolically more active when exposed to treatment. In relation to this, a study reported that treatment of males with highly purified EPA reduced platelet count and platelet size [388]. Park and Harris also demonstrated similar results reporting that EPA, but not DHA reduced platelet size in normal subjects [281] which is consistent with our findings of males and females combined. Therefore our finding in vitro showing that males respond primarily to EPA is supported by previously published studies.

The extent to which in vitro platelet function represents in vivo platelet activity remains unknown. Furthermore, examination of the measures of coagulation factors may shed light on the mechanisms to explain the gender differences noted in this study. The direct clinical implication of this study with reference to gender differences in thrombotic cardiovascular disease is unclear. Our findings, however, raise the possibility of interactions between sex-specific hormones (e.g. testosterone versus estrogen) and LCn-3 PUFA to influence platelet function differentially.

In conclusion, we have shown that DHA, DPA and EPA all effectively reduced in vitro platelet aggregation to the same extent in females, while in males only EPA effectively reduced in vitro platelet aggregation. In vivo studies examining the effects of dietary supplementation with various LCn-3 PUFA on platelet aggregation in males versus females merit investigations. Given the importance of platelets in vascular hemostasis and its role in the pathogenesis of CVD, there is a need for further research directed towards an improved understanding of gender differences in platelet physiology, platelet function and platelet reactivity and the reasons for differential responsiveness to various LCn-3 PUFA.
Chapter Four: Acute dietary supplementation with EPA and DHA rich oils on ex vivo platelet aggregation in male and female subjects

The content of this chapter is covered by the following publications:


Thesis objectives addressed in this chapter:

**Objective 2**: To compare the efficacy of acute dietary supplementation with EPA and DHA rich oils on ex vivo platelet aggregation in healthy human subjects and males vs. females (Section 4.1)

**Objective 3**: To investigate the effects of acute supplementation with EPA or DHA rich oils on circulating platelet microparticle levels and procoagulant activity with respect to platelet aggregation and gender in healthy human subjects (Section 4.2).
4.1. Objective 2

4.1.1. Abstract
Increased platelet aggregation is a major risk factor for heart attacks, stroke and thrombosis. Long chain omega-3 fatty polyunsaturated acids (LCn-3PUFA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) reduce platelet aggregation; however studies in the published literature involving EPA and/or DHA supplementation have yielded equivocal results. Recent in vitro studies have demonstrated that inhibition of platelet aggregation by LCn-3PUFA is gender specific. We examined the acute effects of dietary supplementation with EPA or DHA rich oils on platelet aggregation in healthy male and females. A blinded placebo controlled trial involving 15 male and 15 female subjects was conducted. Platelet aggregation was measured at 0, 2, 5 and 24 hours post supplementation with a single dose of either a placebo or EPA or DHA rich oil capsules. The relationship between LCn-3PUFA and platelet activity at each time point was examined according to gender vs. treatment. EPA was significantly the most effective in reducing platelet aggregation in males at 2, 5 and 24 hours post supplementation (-11%, -10.6%, -20.5% respectively) whereas DHA was not effective relative to placebo. In contrast, in females, DHA significantly reduced platelet aggregation at 24 hours (-13.7%) while EPA was not effective. An inverse relationship between testosterone levels and platelet aggregation following EPA supplementation was observed. Interactions between sex hormones and omega-3 fatty acids exist to differentially reduce platelet aggregation. For healthy individuals, males may benefit more from EPA supplementation while females are more responsive to DHA.

4.1.2. Introduction
Platelets play a primary role in clot development and wound healing, but are also involved in the pathological processes of thrombus formation and blood vessel occlusion. Propensity towards increased platelet aggregation has been shown to be a risk factor for heart attack, stroke, arterial and deep vein thrombosis (DVT) [15, 17]. Indeed high risk individuals such as diabetics and hypertensive patients exhibit increased platelet aggregation [439, 440]. Current pharmacological strategies for the inhibition of platelet aggregation, although effective, present limitations and are not
without adverse effects [147]. Thus there has been increasing interest in the use of non-pharmacological anti-platelet agents, in particular supplementation with long chain omega-3 fatty polyunsaturated acids (LCn-3PUFA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA). Epidemiological and experimental studies have reported a reduction in thrombotic events as well as an overall cardio-protective role of LCn-3PUFA [165, 229, 424, 426]. However it is still unclear which forms of LCn-3PUFA are most beneficial.

Most supplementation studies have been performed using fish oil, the main dietary source of LCn-3PUFA, which contains both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA compete with arachidonic acid (AA) for the 2-acyl position of platelet membrane phospholipids and as a substrate for cyclo-oxygenase, thus reducing the formation of the pro-aggregatory eicosanoid, thromboxane A2 (TXA2) [292]. However, fish oil supplements contain varying amounts of EPA and DHA, it is not clear whether the cardiovascular benefits of LCn-3PUFA are due to EPA or DHA or whether both are equally beneficial. In addition, studies examining the specific effects of EPA and DHA on platelet aggregation have reported conflicting results [280, 282, 283, 285]. We have recently demonstrated that inhibition of platelet aggregation in vitro by treatment with LCn-3PUFA is gender specific, where platelets from males exhibit less aggregation in the presence of EPA, whereas those from females are more inhibited by DHA [441]. These findings made basis for conducting further clinical trials on ex vivo platelet aggregation following dietary supplementation with EPA or DHA rich oils. If such differential responses also occur ex vivo, this may explain some of the previous discrepancies between other studies. The aim of the present investigation was thus to determine if a similar gender bias exists in platelet aggregation in the 24 h following dietary supplementation with a single dose of EPA or DHA rich oils.

4.1.3. Study Design and Methods

4.1.3.1. Participants

This study was a blinded placebo controlled intervention trial. A total of 30 healthy participants completed the study; 15 males and 15 females, recruited from the general community of Newcastle, NSW, Australia. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose
tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m²; taking aspirin, anti-platelet medication or non-steroidal anti-inflammatory drugs. Participants were also excluded if they had consumed fish oil supplements or consumed more than two seafood servings/week for the previous 6 weeks. Participants were asked to complete a medical questionnaire, 24 hour food recall and follow a diet low in tomatoes and seafood at least a week prior to the study day. All participants provided written informed consent according to governmental regulations concerning the ethical use of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia (H-2008-0419) and has been registered in the Australian New Zealand Clinical Trials Registry (ACTRN12609000164291). The study was conducted in accordance with The Declaration of Helsinki. All work was conducted in the research unit at the Medical Sciences Building, University of Newcastle. All participants attended the research unit on 3 separate occasions to consume a different supplement on each occasion with a minimum one week washout period in between. Participants were blinded to consume a single dose of 2 x 1g capsules containing either placebo (Sunola oil), or EPA rich oil (EPAX 5510 TG/N) providing 1g EPA with an EPA/DHA ratio of 5:1, or DHA rich oil (EPAX 1050 TG/N) providing 1g DHA with an EPA/DHA ratio of 1:5. During each visit, a fasting blood sample was collected prior to supplementation, then 2, 5 and 24 hours following supplementation. The participants remained in the research unit for the first 5 hours of the trial, and were then asked to limit physical activity until the final 24 h sample was collected.

4.1.3.2. Platelet aggregation assays

Platelet function was the primary outcome measured. Venous blood was collected into vacutainer tubes containing 3.2% sodium citrate and analyzed 20 minutes after collection. Platelet aggregation and lag time (time taken to initiate aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA) according to the method of Cardinal and Flower [411]. Whole blood (500 µL) was diluted with an equal volume of phosphate-buffered saline. Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5µg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. Reduction of platelet aggregation at 2, 5 and 24 hours post
supplementation was determined by calculating the change in AUC from blood samples collected post supplementation with pre-supplementation baseline aggregation value and expressed as a percentage change. Measurement variation was minimized by having the same trained technician process all samples using the same equipment.

4.1.3.3. Blood analysis
Fasting blood was collected into EDTA and lithium heparin tubes for analysis of full blood count and hormonal levels (testosterone, oestradiol), respectively. Samples were analysed by Hunter New England Area Health Pathology Services (NSW, Australia) using standard analytical techniques.

4.1.3.4. Statistical analysis
Statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago). Sample size was calculated using means, standard deviation and power calculations from our previous data for comparison of platelet aggregation between males and females using a two-tailed t-test, an alpha of 0.05 and a power of 0.90. All data are presented as mean ± SEM. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test variables within gender and LCn-3PUFA group. Statistical significance was assessed using one way ANOVA. Comparisons between gender groups and different LCn-3PUFA groups were made with general linear models using univariate analysis and post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

4.1.4. Results
4.1.4.1. Baseline demographics
All 30 participants completed each treatment group (placebo, n=30; EPA, n=30; DHA, n=30). Significant differences in the baseline characteristics were observed between the male and female participants. Females in the study were older and majority were of post-menopausal age. Males had significantly greater BMI (kg/m$^2$), longer lag time (seconds) and higher levels of testosterone. Females had significantly higher platelet count, higher levels of oestradiol, as well as significantly greater
baseline platelet aggregation than males (Table 4-1). An inverse relationship was observed between aggregation and lag time ($r = -0.799$, $P<0.001$) and a positive relationship was observed between aggregation and platelet count ($r = 0.224$, $P<0.05$). Testosterone levels were inversely correlated with platelet count while oestradiol levels were positively correlated with platelet count (Table 4-2).

**Table 4-1. Characteristics of study participants**

<table>
<thead>
<tr>
<th></th>
<th>Combined (n = 30)</th>
<th>Male (n = 15)</th>
<th>Female (n = 15)</th>
<th>P-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43.7 ± 1.5</td>
<td>40.1 ± 2.1</td>
<td>47.4 ± 1.9</td>
<td>0.014</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.1 ± 0.6</td>
<td>27.6 ± 0.6</td>
<td>24.6 ± 0.8</td>
<td>0.006</td>
</tr>
<tr>
<td>Platelet count (x 10$^9$/L)</td>
<td>256 ± 4.8</td>
<td>237 ± 6.1</td>
<td>275 ± 6.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Baseline Aggregation (AUC)</td>
<td>56.4 ± 1.5</td>
<td>53.1 ± 2.4</td>
<td>59.7 ± 1.9</td>
<td>0.037</td>
</tr>
<tr>
<td>Lag time (sec)</td>
<td>60.4 ± 0.03</td>
<td>68.0 ± 0.06</td>
<td>52.7 ± 0.04</td>
<td>0.043</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>7.8 ± 0.78</td>
<td>14.7 ± 0.56</td>
<td>1.06 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oestradiol (pmol/L)</td>
<td>144.2 ± 21.5</td>
<td>75.0 ± 7.4</td>
<td>213.4 ± 39.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a Values are mean ± SEM; b P-values were obtained using independent samples $t$-test for males vs. females.

**Table 4-2. Correlations between characteristics and platelet aggregatory response**

<table>
<thead>
<tr>
<th></th>
<th>Lag time (sec)</th>
<th>Platelet count (x 10$^9$/L)</th>
<th>Aggregation at 24 hrs EPA</th>
<th>Aggregation at 24 hrs DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline aggregation (AUC)</td>
<td>-.799 b</td>
<td>.224 b</td>
<td>-.054</td>
<td>-.600 b</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>.181</td>
<td>-.342 b</td>
<td>-.443 b</td>
<td>.210</td>
</tr>
<tr>
<td>Oestradiol (pmol/L)</td>
<td>-.222 b</td>
<td>.263 b</td>
<td>.286</td>
<td>-.008</td>
</tr>
</tbody>
</table>

a Values are bivariate correlation and Pearson coefficient; b Values indicate significance at $P<0.05$
4.1.4.2. Platelet aggregation

EPA and DHA rich oils reduced platelet aggregation over 24 hours relative to placebo. EPA was significantly the most effective in reducing platelet aggregation at 2, 5 and 24 hours post-supplementation by -3.6% (P = 0.001), -8.8% (P < 0.001) and -13.3% (P = 0.006) respectively. DHA was not effective at 2 and 5 hours but equally effective as EPA at 24 hours post-supplementation with -11.9% reduction in aggregation relative to placebo (P = 0.016) (Figure 4-1). When the aggregation data was separated into male and female groups, the aggregation response pattern was divided between the male and female subjects. EPA was highly effective in males with reductions in platelet aggregation at 2 (-11%, P = 0.001), 5 (-10.6%, P = 0.003) and 24 hours (20.5%, P = 0.008) respectively, whereas DHA was not significantly effective relative to placebo (Figure 4-2). In contrast, DHA was significantly effective in reducing platelet aggregation at 24 hours (-13.7%) in females while EPA was not effective relative to placebo (Figure 4-3). In both treatment groups, the maximal effect observed was at 24 hours following supplementation. In males, the mean lag time increased by 29.5% (60 vs. 79 secs) at 24 hours post EPA supplementation (data not shown). In addition, an inverse relationship was observed between platelet aggregation activity at 24 hours post EPA supplementation with testosterone levels, while an inverse relationship was observed between platelet aggregation activity at 24 hours post DHA supplementation with baseline platelet aggregation value (Table 5-2).
Figure 4-1. Effect of supplementation on platelet aggregation over time in males and females. Results are mean AUC % change using one-way ANOVA with post hoc Tukey tests; values without a common symbol within a time point are significantly different. \( P<0.05 \).

Figure 4-2. Effect of supplementation on platelet aggregation over time in males. Results are mean AUC % change using one-way ANOVA with post hoc Tukey tests; values without a common symbol within a time point are significantly different. \( P<0.05 \).
Figure 4-3. Effect of supplementation on platelet aggregation over time in females. Results are mean AUC % change using one-way ANOVA with post hoc Tukey tests; values without a common symbol within a time point are significantly different. $P<0.05$.

### 4.1.5. Discussion

In the present study, we have shown that gender-specific responses exist in platelet aggregation in the 24 h following dietary supplementation with a single oral dose of EPA or DHA rich oil capsules. These data confirm our previous observations that treatment of platelets with EPA or DHA in vitro inhibits platelet aggregation in a gender-specific manner [441]. Previously published studies concerning the effects of EPA and/or DHA supplementation on platelet aggregation have been conducted in mixed gender groups or males only resulting in conflicting results against DHA [208, 280, 282]. Similarly, in the present study the combined male and female population indicated that only EPA was effective in reducing aggregation at 2, 5 hours from time of supplementation, whereas both DHA and EPA were equally effective at 24 hours, reducing platelet aggregation to a similar extent when the entire cohort was considered. However, further analysis revealed that the aggregation response pattern
was strictly divided between the male and female subjects, where the anti-aggregatory effect of EPA was only evident in male subjects while the anti-aggregatory effect of DHA was unique to female subjects. This novel finding suggests that the controversies surrounding results from previously reported studies may be resolved by reanalysing the data based on gender.

The underlying cardio protective effects of EPA and DHA on platelet function is that these fatty acids compete with AA for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level to modulate the production of pro-thrombotic eicosanoids. AA is the precursor of the prostanoids of the 2-series (PGI$_2$ (prostacyclin), TXA$_2$), whereas EPA is the precursor of prostanoids of the 3-series (PGI$_3$ and TXA$_3$) which are biologically less active [292]. Therefore the results of these changes in eicosanoid production are vasodilatation and inhibition of platelet aggregation. However, the underlying anti-aggregatory effects of LCn-3PUFA on platelet function have been more attributable to EPA as it is preferentially incorporated into platelets [205] compared with DHA. Interestingly, previous reports have shown that in males, EPA incorporation into plasma lipids and platelet phospholipids increased 2-5 fold higher than DHA following a fish diet or fish oil supplementation [205, 294, 350]. The observed effects of EPA in our study may be owing to the possibility that EPA is more effectively incorporated into platelets in males than females. Furthermore, analysis of plasma fatty acids in our study cohort also revealed that circulating DHA levels were increased only in females subjects 24 hours following DHA supplementation (unpublished data).

Gender differences observed could also be due to the interaction between sex hormones to influence circulating levels of EPA/DHA and thus their mechanisms of action. It has been suggested that the effects of DHA are surface mediated [350, 442] thus possibly acting upon surface cell adhesion molecules (CAM) or receptors. Indeed LC n-3 PUFA can modulate the interaction between hormones and their receptors [443] and DHA has been reported to decrease specific binding to TXA$_2$/prostaglandin H$_2$ receptors [444]. Several studies have also reported that testosterone regulates the expression of platelet TXA$_2$ receptors, thereby increasing vascular responses to TXA$_2$ [331, 332]. Ajayi et al demonstrated that in healthy males, administration of testosterone indeed increased platelet TXA$_2$ receptor density.
and aggregation responses [333]. The reported association between testosterone with increased platelet TXA₂ receptor density is consistent with our finding that DHA is not effective in males as testosterone may counteract the inhibitory effects of DHA. Inhibition of platelet aggregation can also be achieved by reducing levels of circulating CAM thus reducing endothelial activation. It has also been demonstrated that the ability of PUFA to reduce levels of CAM increased with the degree of unsaturation, thus DHA was the most potent inhibitor of endothelial activation [386]. In addition, a study has reported that DHA content of granulocytes is negatively associated with ICAM-1 levels, while granulocyte content of EPA did not correlate with ICAM-1 or VCAM-1 levels [334]. Interestingly, the authors also concluded that the effects of LCn-3PUFA on CAM may depend on gender and LCn-3PUFA dose. Furthermore, P-selectin has been reported to be inversely associated with oestrogen levels [325], indeed females are reported to have significantly lower levels of CAM expression [445] and higher levels of DHA [348]. Higher synthesis and/or retention of DHA in females compared to males may explain the observed greater inhibition of platelet aggregation in females, particularly during the child-bearing age.

These data lend strength to the fact that gender is a confounding risk factor for thrombotic disease. Previous studies that have reported the lack of effect of DHA to inhibit platelet aggregation have been conducted in males only [208, 280, 282]. Our results along with the reported literature suggest that these studies merits re-examination in the light of gender differences, and future studies should not be limited to males only.

At baseline, an inverse relationship was observed between lag time and platelet aggregation. The inhibition of platelet aggregation was dependent on lag time as reduced platelet reactivity was associated with increased lag time. Furthermore, lag time progressively increased with EPA supplementation in males in accordance with the continuous decrease in platelet aggregation over time. At 24 hours post EPA, lag time increased by 29.5% in males (60 vs. 79 secs). The increased lag time observed in our study further suggest that EPA may reduce aggregation through weakened or delayed responses to aggregatory agents thereby increasing prothrombin time and bleeding time [295]. Lag time is indicative of platelet responsiveness, an association between platelet responsiveness and cardiovascular deaths has been reported in a study involving healthy males [446]. Males with enhanced platelet reactivity
characterised by shorter lag time, had higher risks of cardiovascular mortality. Our finding that EPA may extend lag time to reduce platelet aggregation in males is indeed a novel and highly significant observation. There has been no reported association between DHA with prothrombin or bleeding time, rather the anti-aggregatory effects of DHA has been reported to be independent of prothrombin fragments [280, 282], which may explain why lag time was not related to the reduced aggregation observed with DHA in female subjects.

Females in our study had significantly higher platelet counts and platelet aggregation. Correlations showed that higher platelet count was associated with higher levels of oestradiol and lower levels of testosterone. Interestingly, our correlations also showed that higher levels of testosterone were associated with a greater reduction in platelet aggregation at 24 hours post EPA supplementation which indeed supports our aggregation data. Of further interest, our correlation data also showed that higher baseline aggregation was associated with greater reductions in platelet activity at 24 hours post DHA supplementation. Females in our study had 16 % higher baseline aggregation than males, and responded only to DHA at 24 hours.

The mechanisms by which sex hormones influence the platelet aggregatory response to EPA and DHA cannot be ascertained from the present study. However, regardless of the mechanisms by which the gender differences are expressed, our findings clearly indicate an interaction between sex hormones and supplementation with EPA or DHA to reduce platelet activity in healthy males and females. It should be noted that our present study is reporting the immediate effects (2-24 hrs) of a single dose supplement; the conversion of EPA to DHA though present is very inefficient where studies have suggested that the overall conversion rate is between 0-9%. Furthermore, interconversion between EPA and DHA have only been reported in studies with a minimum 2 week supplementation period [201]. We have demonstrated for the first time a gender-specific response in ex vivo platelet aggregation following a single dietary dose of EPA or DHA rich oils. The distinctive gender-specific, inhibitory response pattern observed in our study suggests that males may benefit more from EPA supplementation while females are more responsive to DHA. It should also be noted that our study population was comprised of healthy
individuals with no history of platelet abnormalities. In addition to the female population, it is possible that DHA may also be effective in modulating platelet activity in situations where platelets are hyperactive such as individuals with type 2 diabetes [202]. Future studies examining EPA and/or DHA in males versus females should be extended to populations or individuals with abnormal platelet function when examining the association between dietary LCn-3PUFA and biomarkers of thrombotic disease risk.

4.2. Objective 3

4.2.1. Abstract
Dietary supplementation with omega-3 fatty acids has been associated with reduced incidence in thrombotic events. In addition, administration of n-3PUFAs has been shown to rectify elevated platelet microparticle (MP) number and procoagulant activity in post myocardial infarction patients. However, it is unknown whether supplementation can alter these parameters in healthy individuals, and if such effects are immediate or require long term supplementation. We have previously demonstrated a gender-specific effect of LCn-3PUFA supplementation on platelet aggregation in healthy human subjects. Here we extend these findings to include the acute effects of supplementation with EPA or DHA rich oils on circulating MP levels and activity in healthy subjects. A placebo controlled trial was conducted in healthy males and females (n=30). MP activity, MP levels and platelet aggregation were measured at 0 and 24 hours post supplementation with either a placebo or EPA or DHA rich oil. Both EPA and DHA effectively reduced platelet aggregation at 24 hours post supplementation relative to placebo (-13.3%, $P=0.006$ and -11.9%, $P=0.016$ respectively), but only EPA reduced MP activity (-19.4%, $P=0.003$). When grouped by gender, males showed a similar reduction in both platelet aggregation and MP activity (-20.5%, $P = 0.008$; -22%, $P=0.008$) following EPA, while females showed significantly reduced platelet aggregation (-13.7%, $P=0.04$) but not MP activity after DHA only. EPA and DHA exert gender-dependent effects on platelet aggregation and platelet MP activity, but not on MP levels. With respect to thrombotic disease risk, males may benefit more from EPA supplementation.
4.2.2. Introduction

Microparticles (MP) are small phospholipid-rich, membrane bound fragments shed from stimulated or apoptotic cells. Derived from various cell types, they differ in their antigenic composition and functional properties, which are specific to their cellular origin and the cellular processes triggering their formation [447]. MPs circulate at low levels in the bloodstream of healthy individuals, but are increased in a variety of pathological states. The most abundant are the platelet derived MP, less than 1.0um in diameter and constituting approximately 70% to 90% of all circulating MPs [448]. Platelet MPs were first described by Wolf as tiny membrane fragments known as ‘platelet dust’ that were released upon platelet activation but possessing comparable procoagulant activity as activated intact platelets [449]. In a resting platelet, the negatively charged phospholipids are distributed in the inner membrane layer. When platelets become activated the nature of the membrane is disrupted and these phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are relocated to the outer membrane and exposed [115]. Exposure of PS provides a negatively charged surface to bind coagulation factors and allow the assembly of the prothrombinase complex where prothrombin is cleaved into thrombin [450]. With respect to activated platelets, MPs are formed from the phospholipid rich outer platelet membrane thus providing additional phospholipid surface for assembly of various enzyme complexes of the coagulation cascade [90]. Since the discovery of MP, further investigation has revealed diverse roles in coagulation, cellular interactions and cell signalling. They are able to transfer their procoagulant potential to target cells [451], bind to the surface antigens of other cells and by doing so induce intracellular signalling pathways [114]. With further insight, platelet MPs are now acknowledged as bioactive vascular effectors, a storage pool for procoagulant phospholipids and a key modulator of the hemostatic balance. Indeed elevated levels of circulating platelet MPs are observed in diseases of vascular involvement and high platelet aggregation activity [452] and platelet aggregation which is initiated by platelet activation is an early event in the development of thrombosis [180]. Hence platelet MPs are now considered as one of the reliable markers of platelet hyperactivity in determining the risk of cardiovascular and thrombotic diseases.
Dietary supplementation with long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) has been associated with reduced incidences in thrombotic events and significant cardiovascular risk reduction [165, 229, 424, 426]. Many of the cardio protective effects are attributed to the incorporation of LCn-3PUFAs into the phospholipid membranes of platelets and endothelial cells [453]. Here EPA and DHA compete with arachidonic acid (AA) as a substrate for cyclo-oxygenase to reduce formation of pro-aggregatory eicosanoids [231, 275, 283, 292, 370, 419]. Indeed the platelet anti-aggregatory effects of LCn-3PUFA have been reported [275, 283] in term long supplementation studies. We have also recently demonstrated that LCn-3PUFAs effectively reduces platelet aggregation over 24 hours in healthy human subjects [454]. Furthermore, the anti-aggregatory effects observed were dependent on gender and the different concentration ratios of the LCn-3PUFAs (EPA vs. DHA).

Whether supplementation with LCn-3PUFA affects platelet MP generation or MP activity in healthy male and female subjects have not been studied. Furthermore it is also not known whether circulating platelet MP levels correlate with MP activity in healthy individuals. The aim of the present investigation was thus to investigate the effects of acute supplementation with EPA or DHA rich oils on circulating platelet MP levels and procoagulant activity with respect to platelet aggregation and gender in healthy human participants.

4.2.3. Study design and methods

4.2.3.1. Participants

This study was a blinded placebo controlled intervention trial. A total of 30 healthy participants completed the study; 15 males and 15 females, recruited from the general community of Newcastle, NSW, Australia. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m²; taking aspirin, anti-platelet medication or non-steroidal anti-inflammatory drugs. Participants were also excluded if they had consumed fish oil supplements or consumed more than two seafood servings/week. Participants were asked to complete a medical questionnaire, 24 hour food recall and follow a diet low in tomatoes and seafood prior to the study day. All participants provided written informed consent according to governmental regulations concerning the ethical use
of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia (H-2008-0149) and has been registered in the Australian New Zealand Clinical Trials Registry (ACTRN12609000164291). The study was conducted in accordance with The Declaration of Helsinki. All work was conducted in the research unit at the Medical Sciences Building, University of Newcastle. All participants attended the research unit on 3 separate occasions to consume a different supplement on each occasion with a minimum one week washout period in between. Participants were blinded to consume a single dose of 2 x 1g capsules containing either placebo (Sunola oil), or EPA rich oil (EPAX 5510 TG/N) providing 1g EPA with an EPA/DHA ratio of 5:1, or DHA rich oil (EPAX 1050 TG/N) providing 1g DHA with an EPA/DHA ratio of 1:5. During each visit, a fasting blood sample was collected prior to supplementation and 24 hours following supplementation. The participants were asked not to consume foods containing any seafood or tomato products and limit physical activity until the final 24 h sample was collected.

4.2.3.2. Blood analysis

Venous blood was collected into vacutainer tubes containing 3.2% sodium citrate following a < 10 hr fast. Whole citrate blood was analysed 20 min after collection for platelet aggregation assays. For measurement of PMP activity and PMP numbers, citrate blood was centrifuged at 3000 x g for 10 min to obtain platelet free plasma and aliquots were stored at -80°C until further analysis. Samples were thawed at 37°C within 15 min prior to testing. Fasting blood was collected into EDTA and lithium heparin tubes for analysis of full blood count and hormonal levels (testosterone, oestradiol), respectively. Samples were analysed by Hunter New England Area Health Pathology Services (NSW, Australia) using standard analytical techniques.

4.2.3.3. Platelet aggregation assays

Platelet aggregation and lag time (time taken to initiate aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA) according to the method of Cardinal and Flower [411]. Whole blood (500 μL) was diluted with an equal volume of PBS. Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5μg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated.
Reduction of platelet aggregation was determined by calculating the change in AUC from blood samples collected 24 hr post supplementation with baseline aggregation values and expressed as a percentage change. Measurement variation was minimized by having the same trained technician process all samples using the same equipment.

4.2.3.4. Measurement of microparticle activity
The procoagulant activity of microparticles was measured using the Zymuphen MP-Activity ELISA kit (HYPHEN BioMed, Neuville-sur-Oise, France) following the manufacturer’s instructions. In this assay Annexin V is used for capturing MPs expressing phosphatidylinerine (PS) (procoagulant activity) where MP activity in the sample was determined by the binding to Annexin V in the wells. The unbound particles were washed away and Factor Xa-Va mixture and prothrombin was added. Exposure of phospholipid surface of the Annexin V bound microparticles allowed the activation of prothrombin to thrombin. Thrombin was measured via cleavage of a chromogenic thrombin substrate and the absorbance read at 405 nm with a correction at 690 nm. The amount of thrombin generation is directly related to the phospholipid concentration in the plasma. The results were compared to a standard curve of known MP concentration expressed in nM phosphatidylinerine (PS) equivalent. All samples were analysed in duplicate and samples from one participant were determined in the same series, to avoid bias due to assay variability. Only replicates with a CV < 15 were used in the final analysis.

4.2.3.5. Flow cytometry
Platelet MP was identified and quantified by flow cytometry with specific platelet (CD41a), surface markers. All reagents and solutions used were sterile and filtered (0.2 µm filter). A 30 µl aliquot of platelet-free plasma was incubated at room temp for 15 min with 10 µL of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA) and 10 µL of Annexin V- FITC (BD PharMingen, San Diego, CA, USA) as a marker of activation/ PS expression. The sample was incubated for 30 min at room temp then diluted with 500µL of PBS and stained with 30 µL fluorospheres (Flow-Count, Beckman Coulter Inc., CA USA). A known number of 10 µm enumeration beads (Flow-count Fluorospheres; Beckman Coulter, Fullerton, CA, USA) were added prior to analysis and data was analysed using a BD FACS Canto flow cytometer and CXP software (BD Biosciences, CA, USA) according to a standardised method.
The MP gate, based on a particle size of <1.0 µm, was used for identification of MP. Events in the MP gate were assessed for labelling with antibody positive events to distinguish true events from background noise. The number of CD41+ Annexin V bound MP were identified as MP expressing procoagulant activity. Microparticles were quantified using a modified Combes method [416] and enumerated by using the formula:

\[(MP \text{ count/bead count}) \times (\text{bead concentration}/\mu L) = MP \text{ count }/\mu L\]

**4.2.3.6. Statistical analysis**

Statistical analyses were performed using PASW Statistics software (version 18, SPSS Inc., Chicago). Sample size was calculated using means, standard deviation and power calculations from our previous data for comparison of platelet aggregation between males and females using a two-tailed t-test, an alpha of 0.05 and a power of 0.90. All data are presented as mean ± SEM. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test variables within gender and LCn-3PUFA group. Statistical significance was assessed using one way ANOVA and the t-test for paired data. Comparisons between gender groups and different LCn-3PUFA groups were made with general linear models using univariate analysis and post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

**4.2.4. Results**

**4.2.4.1. Baseline demographics**

All 30 participants completed each treatment (placebo, \(n=30\); EPA, \(n=30\); DHA, \(n=30\)). Significant differences in the baseline characteristics were observed between the male and female participants. Females in the study were older and majority were of post-menopausal age. Males had significantly greater BMI (kg/m²), longer lag time (seconds) and higher levels of testosterone. Females had significantly higher platelet count, higher levels of oestradiol, as well as significantly greater baseline platelet aggregation than males. Despite this, there were no significant gender differences between platelet MP activity or CD41+ Annexin V bound MP (Table 4-3).
### Table 4-3. Characteristics of study participants

|                          | Combined                          | Male                        | Female                       | P-value  
|--------------------------|-----------------------------------|-----------------------------|------------------------------|----------  
|                          | (n = 30)                          | (n = 15)                    | (n = 15)                     |          2  
| Age (years)              | 43.7 ± 1.5                        | 40.1 ± 2.1                  | 47.4 ± 1.9                   | 0.014    1  
| BMI (kg/m²)              | 26.1 ± 0.6                        | 27.6 ± 0.6                  | 24.6 ± 0.8                   | 0.006    1  
| Platelet count (x 10⁹/L) | 256 ± 4.8                         | 237 ± 6.1                   | 275 ± 6.2                   | <0.001   1  
| Platelet aggregation (AUC) | 56.4 ± 1.5                        | 53.1 ± 2.4                  | 59.7 ± 1.9                   | 0.037    1  
| Lag time (sec)           | 60.4 ± 0.03                       | 68.0 ± 0.06                 | 52.7 ± 0.04                  | 0.043    1  
| MP activity (nM)         | 23.05 ± 1.1                       | 23.0 ± 1.76                 | 23.1 ± 1.37                  | NS       1  
| CD41+ Ann-V (MP/µL)      | 13.7 (11.6-15.7)                  | 14.0 (11.3-16.7)           | 13.4(10.2-16.6)             | NS       1  
| Testosterone (nmol/L)    | 7.8 ± 0.78                        | 14.7 ± 0.56                 | 1.06 ± 0.08                  | <0.001   1  
| Oestradiol (pmol/L)      | 144.2 ± 21.5                      | 75.0 ± 7.4                  | 213.4 ± 39.8                | 0.001    1  

1 Values are mean ± SEM; 2 P-values were obtained using independent samples t-test for males vs. females; 3 Values reported are x 10⁹; lower and upper quartiles are reported in brackets; BMI, body mass index; MP, microparticle.
### 4.2.4.2. Relationship between platelet and microparticle parameters

In the combined population, platelet aggregation was found to be inversely correlated to lag time \( (r = -0.765, P<0.001) \), and positively correlated to platelet count \( (r = 0.224, P<0.05) \) and microparticle activity \( (r = 0.318, P=0.002) \), but not to the number of CD41+ Annexin V bound MP (Table 4-4). When the data was separated into the gender groups, only males continued to demonstrate the positive correlation between platelet aggregation and MP activity \( (r = 0.456, P=0.002) \) (Figure 4-4). In addition, lag time was inversely correlated to platelet MP activity \( (r = -0.308, P=0.039) \) in this group. In contrast, only the females demonstrated a positive relationship between the number of CD41+ Annexin V bound MP and MP activity \( (r = -0.395, P=0.011) \).

### Table 4-4. Correlations between platelet and MP parameters in the total cohort (n=30)^{1}

<table>
<thead>
<tr>
<th></th>
<th>MP activity</th>
<th>MP number</th>
<th>Lag time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation</td>
<td>( r = 0.318 )</td>
<td>( r = -0.210 )</td>
<td>( r = -0.765 )</td>
</tr>
<tr>
<td></td>
<td>p-value = 0.002</td>
<td>p-value = NS</td>
<td>p-value &lt;0.001</td>
</tr>
<tr>
<td>Lag time</td>
<td>( r = -0.185 )</td>
<td>( r = 0.139 )</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>p-value = NS</td>
<td>p-value = NS</td>
<td></td>
</tr>
<tr>
<td>MP number</td>
<td>( r = -0.196 )</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>p-value = NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1 Results are bivariate correlation and Pearson Coefficient
Figure 4-4. Gender difference in the relationship between the baseline aggregation and MP activity. Results are bivariate correlation and Pearson coefficient for AUC and MP procoagulant activity of baseline sample.

4.2.4.3. Effect of LCn-3PUFA supplementation on platelets and platelet derived microparticles

When the cohort was analysed as a whole, supplementation with either EPA or DHA was effective at reducing platelet aggregation (-13.3 %, $P = 0.006$; -11.9%, $P=0.016$ respectively) relative to placebo (Figure 5-5). However, only EPA rich oil produced a decrease in microparticle activity (-19.4%, $P=0.003$), and neither oil resulted in changes to microparticle numbers (Figure 5-6). When separated by gender, the response to supplementation was differential amongst the male and female subjects. In males, the mean lag time increased by 29.5 % (60 vs. 79 secs) at 24 hours post EPA supplementation. In addition, an inverse relationship was observed between platelet aggregation activity at 24 hours post EPA supplementation with testosterone levels ($r = -0.443$, $P=0.04$). EPA was effective only in males with - 20.5%, ($P = 0.008$) reduction in platelet aggregation, and a similar reduction in MP activity (- 22%, $P= 0.008$), whereas DHA was not significantly effective relative to placebo (Figure 3). In addition, the mean lag time increased by 29.5 % (60 vs. 79 secs). In contrast, DHA was effective in reducing platelet aggregation in the female subjects.
only (-13.7%), but did not significantly affect any of the other parameters. The number of CD41+ Annexin V bound MP was also not affected following either EPA or DHA supplementation in either gender groups.

Figure 4-5. Effect of LCn-3PUFA on platelet aggregation in males and females. Results are mean AUC% change one-way ANOVA with post hoc Tukey tests; values sharing a common symbol (*, †, ‡, ††) are significantly different, P<.05.
Figure 4-6. Effect of LCn-3PUFA on MP activity in males and females. Results are mean MP% change one-way ANOVA with post hoc Tukey tests; values sharing a common symbol (*,†) are significantly different, P<.05.

4.2.5. Discussion

This study demonstrates for the first time that a single dose of EPA rich oil significantly inhibits platelet MP activity in parallel with a reduction in platelet aggregation, while supplementation with DHA rich oils reduces platelet aggregation independent of MP activity. The present finding that EPA and DHA affect platelet derived MP activity differentially strengthens our previous findings of gender-specific platelet aggregation response.

In the combined study population, both EPA and DHA effectively reduced platelet aggregation to a similar extent when compared with placebo. When separated into the male and female groups, the aggregation response pattern was divided between the gender groups such that the anti-aggregatory effects of EPA was owing to the male subjects and the effect of DHA was unique to the female subjects. The demonstration of such a striking response pattern clearly suggested differences in the mechanistic pathways whereby these LCn-3 PUFAs exert their anti-aggregatory effects. Since platelet aggregation is initiated following platelet activation and platelet activation results in the release of procoagulant MP, we studied the effects of
LCn-3PUFA on circulating platelet MP levels and activity. Indeed our study findings indicate platelet MPs as a potential pathway whereby LCn-3PUFAs may differentially modulate the hemostasis.

Our findings clearly demonstrate that a single oral dose of EPA rich oils decreases platelet MP activity in healthy subjects whereas supplementation with DHA rich oils is not effective. EPA significantly reduced platelet MP activity by – 19.4% ($P=0.003$) 24 hr following supplementation relative to placebo. When the MP data was segregated into the male and female groups, a gender biased response was once more observed such that the effect of EPA to reduce MP activity was predominantly owing to the male subjects. In males, there was a -22% ($P=0.008$) reduction in MP activity where in females the effects of EPA were no longer significant relative to placebo. Interestingly, there were no effects on the number of CD41+ Annexin V bound MP following supplementation; indicative that the anti-aggregatory effects of EPA are via the inhibition of the procoagulant MP activity but with no effects on circulating MP levels in plasma. Perhaps this could be considered more favourable given that the mechanism of MP clearance from the circulation is not known in humans. Therefore decreasing levels of circulating MP would not be of further benefit if the clearance rate is rapid in humans. Microparticle clearance has been reported to be rapid in animal studies regardless of treatment. In mice, platelet MP are cleared from the circulation within 30 min following infusion and less than 10 min in rabbits [455]. The implication that endogenous platelet MP are rapidly cleared from the circulation suggests that they are generated continuously to maintain baseline levels as they continue to circulate in healthy individuals in the absence of trauma [448]. In our study, reduction in MP activity but not in MP levels suggests that the new platelet MPs generated following EPA supplementation are less procoagulant, possibly due to the incorporation of EPA into the platelet membrane. The kinetics of the incorporation of LCn-3PUFAs into plasma phospholipids and platelets has been reported in the GISSI-Prevenzione Study. An early and rapid increase of EPA was found in platelets and plasma phospholipids after one week following a low dose of LCn-3PUFA while DHA levels were not affected in platelets [32]. Indeed EPA is preferentially incorporated into platelets [205] compared with DHA. Of further interest, reports have shown that in males, EPA incorporation into plasma lipids and platelet phospholipids increased 2-5 fold higher than DHA.
following fish oil supplementation [205, 294, 350]. Consistent with the finding that MP activity was reduced in parallel with platelet aggregation in males, MP procoagulant activity was positively associated with platelet aggregation in the male population only. Furthermore, lag time (time taken to initiate aggregation) was inversely associated with platelet MP activity and indeed lag time increased by 29.5% (60 vs. 79 s) in males following EPA supplementation. Lag time is reflective of prothrombin time and this is important given that MP activity is initiated following the exposure of PS where prothrombin is cleaved into thrombin [450]. The increased lag time in parallel with the decreased MP activity and platelet aggregation observed in our study suggest a potential pathway whereby EPA may reduce platelet aggregation via incorporation of EPA into PS thus increasing prothrombin time. Indeed EPA has been reported to be incorporated in PS following EPA supplementation in healthy male subjects [456]. It is also interesting to note that Throngren and co-workers have reported that an EPA enriched diet significantly increased prothrombin time and bleeding time in a population of healthy male individuals [295]. The observed effects of EPA in our study may be owing to the possibility that EPA is more effectively incorporated into platelets in males than females, while females are able to retain DHA more efficiently. Furthermore, analysis of plasma fatty acids in our study cohort also revealed that circulating DHA levels were increased only in females subjects 24 hrs following DHA supplementation (unpublished data). Indeed females have a greater capacity to synthesize DHA compared to males [200, 346, 457]. Oestrogens have been shown to increase DHA synthesis, while testosterone have been reported to decrease DHA concentrations [337]. Higher synthesis and/or retention of DHA in females compared to males may explain the observed greater inhibition of platelet aggregation in females.

Gender differences observed in platelet aggregation could also be due to the interaction between sex hormones to influence circulating levels of EPA/DHA and thus their mechanisms of action. It has been suggested that the effects of DHA are surface mediated [350, 442] thus possibly acting upon surface cell adhesion molecules (CAM) or receptors. It has been reported that DHA content of granulocytes is negatively associated with ICAM-1 levels, while granulocyte content of EPA is not correlated with ICAM-1 or VCAM-1 levels [334]. Furthermore, P-
selectin has been reported to be inversely associated with oestrogen levels [325], indeed females are reported to have significantly lower levels of CAM expression [253, 445] and higher levels of DHA [348].

Particularly striking was that a positive relationship was observed between the number of CD41+ Annexin V bound MP and platelet aggregation in the female population. This suggests that in females platelet aggregation is predominately dependant on the level of circulating MPs. Further supporting this observation, circulating MP levels were not affected following EPA supplementation hence platelet aggregation was also not reduced. Females in our study had a higher platelet count which may have also allowed for increased MP generation with respect to increased platelet numbers. MP numbers were not affected following DHA supplementation in females which suggest that DHA reduces platelet aggregation in females independent of MP numbers and activity.

The lack of effect of EPA to reduce platelet aggregation in females is further strengthened by the finding that MP activity was not affected in females following EPA supplementation. Furthermore, DHA was not effective in reducing MP activity or bearing any relationship with baseline MP activity in both male and female subjects. These findings confirm 2 things; 1: DHA acts to reduce platelet aggregation differentially, 2: DHA is not effective to reduce platelet aggregation in males. No other studies have investigated the differential effects of LCn-3PUFA supplementation on platelet MP in healthy individuals; however combined EPA/DHA supplementation in patients with previous myocardial infarction has been shown to significantly reduce both the number and pro-coagulant activity of platelet derived MPs over a 12 week period [389]. It is possible that while MP activity can be immediately reduced via incorporation of LCn-3PUFA into the platelet membrane, the production of MPs may take longer to stabilise. Alternatively, the increased levels of MPs in this group of patients may be the result of a pathological condition that simply does not exist in a healthy cohort, and thus treatment with LCn-3PUFA will not reduce MP levels below a normal range. We are presently investigating this hypothesis with a longer intervention trial.
The mechanisms by which acute supplementation with EPA/DHA rich oil alters platelet aggregation or microparticle activity are not clear. Since EPA/DHA levels were significantly increased following supplementation for 24 hours, it is likely that at least some of EPA/DHA may have made their way into platelet membranes via exchange process and/or direct uptake. To date no study has examined the acute effects of EPA/DHA supplementation on fatty acid incorporation into platelet membranes. This is an important point that warrants examination in future studies. Though the precise mechanism involved in the protection against thrombotic disease risk cannot be elucidated, our study findings clearly indicate that both EPA and DHA reduce platelet aggregation in males and females differentially. The novel finding that EPA inhibits MP activity in male subjects further strengthens our previous findings of the gender-specific platelet aggregation response of EPA vs. DHA. Hence our study suggests that supplementation with EPA but not DHA inhibits platelet MP activity in parallel with a reduction in platelet aggregation in a gender-specific manner. Our finding that EPA rich oils inhibit procoagulant MP activity is important given that thrombosis remains one of the leading causes of mortality in developed counties and platelet MP are now recognised as pathogenic markers of thrombotic disease.
Chapter Five: Long term effects of dietary supplementation with EPA or DHA-rich oils on platelet and coagulant activity in male vs. female subjects

The content of this chapter is covered by:

Phang, M., Lincz, L & Garg & M.L; ‘Differential effects of eicosapentaenoic and docosahexaenoic acid on platelet aggregation and hemostatic markers in male versus female subjects’ (Under review).

Phang, M., Scorgie, F.E, Seldon, M.,Garg, M.L &Lincz, L; ‘Reduction of prothrombin and Factor V levels following supplementation with omega-3 fatty acids is gender-dependant: a randomised controlled study’ (Under review).

Thesis objectives addressed in this chapter:

Objective 4: To examine the effects of long-term dietary supplementation with EPA or DHA rich oils on ex vivo platelet aggregation in healthy male versus female subjects. (Section 5.1)

Objective 5: To compare the effects of long-term dietary supplementation with EPA and DHA rich oils on plasma fatty acid profiles, procoagulant activity, coagulation factors and biochemical hemostatic markers of platelet aggregation in males vs. females. (Section 5.2)
5.1. Objective 4

5.1.1. Abstract
Although consumption of n-3 fatty acids (LCn-3PUFA: eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) have been reported to reduce platelet aggregation however the available evidence on this is equivocal. We have previously demonstrated that the acute (24h) effects of LCn-3PUFA supplementation on platelet aggregation are gender dependent. We aimed to determine if this gender bias is maintained during long term LCn-3PUFA supplementation and whether this translates to other hemostatic markers. A double-blinded randomised place controlled trial was conducted in 94 healthy men and women. Platelet aggregation, thromboxane B₂ (TXB₂), P-selectin (P-sel), von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) were measured at baseline and 4 weeks post-supplementation with EPA or DHA-rich oil capsules. The relationship between LCn-3PUFA and platelet activity was examined according to gender vs. treatment. EPA and DHA effectively reduced platelet aggregation following 4 weeks supplementation relative to placebo (-11.8%, $P=0.016$ and -14.8%, $P=0.001$ respectively). In males, only EPA reduced platelet aggregation (-18.4%) compared to placebo ($P=0.005$) and the female group ($P=0.011$). In contrast, in females only DHA reduced platelet aggregation (-18.9%) compared to placebo ($P=0.001$) and the males ($P=0.017$). Significant gender x treatment interactions were also observed between hemostatic markers and uptake of LCn-3PUFA to reduce platelet aggregation differentially in men compared to women. With respect to thrombotic disease risk, men are more likely to benefit from supplementation with EPA, while women are more responsive to DHA.

5.1.2. Introduction
Fatty fish and marine oils that are rich sources of long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA), have sparked considerable interest in epidemiological and clinical studies, suggesting favourable effects for prevention of atherosclerotic and thrombotic disease. The early observations that LCn-3PUFA promotes cardio-protection were first demonstrated in the 1970s by Dyerberg and Bang in a series of prospective studies on Greenland Eskimos [458]. These studies
revealed that the rarity of ischemic heart disease and decreased thrombotic tendency in this population was linked to their consumption of a seafood diet high in the LCn-3PUFAs: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The formation of occlusive thrombi resulting in cardiovascular complications is generally a platelet-dependent phenomenon. This process is initiated when platelets adhere to subendothelial structures of injured blood vessels, release vasoactive substances, and aggregate, resulting in the formation of platelet-rich thrombi. These sequential primary hemostatic events are dependent and mediated by an interplay of cellular adhesion molecules and proteins such a P-selectin (P-sel) and von Willebrand factor (vWF); pro-aggregatory factors including thromboxane (TX) A2 as well as coagulation factors of secondary hemostasis [51]. Inhibition of these pro-coagulant elements can facilitate thrombus dissolution via the fibrinolytic system [459]. Subsequently, degradation of the fibrin clot also plays a pivotal role in the control of thrombus development, and abnormally elevated levels of the fibrinolysis inhibitor, plasminogen activator inhibitor-1 (PAI-1) have been associated with some instances of thrombotic disease [460].

LCn-3PUFA provide a remarkable contribution to the physical properties of biological membranes and the ability to modulate the activity of membrane-associated proteins [217]. In addition, the incorporation of LCn-3PUFA in cell membranes also modifies eicosanoid production. The accumulation of EPA and DHA into platelet phospholipids provides a partial replacement of the omega-6 arachidonic acid (AA) to compete as a substrate for the cyclooxygenase (COX) enzymes to catalyse the biosynthesis of TXA3 and subsequently reduced conversion to TXA2 [230]. However the inverse relationship between LCn-3PUFA intake and platelet aggregation has not been a consistent finding. While some dietary intervention studies have reported that LCn-3PUFA supplementation reduces formation of TXA2 [275, 292, 373], prolongs bleeding time [461], reduces platelet count [388] and is inversely associated with expression of cellular adhesion molecules and plasma levels of several coagulation factors [380], other studies have yielded equivocal evidence for their effectiveness against these biomarkers of hemostasis [280-285, 462]. Disparate findings concerning the effects of LCn-3PUFA on platelet function may be explained by gender-dependant differences in platelet
aggregation in response to EPA or DHA. Despite the fact that fish and fish oils contain EPA and DHA in variable amounts, few well-designed studies in humans have assessed their individual effects. In the available fish oil and platelet aggregation literature, wide variability in terms of dosage and concentration ratios of EPA: DHA are apparent. This variable, coupled with the non-modifiable factor of gender, may likely explain the inconsistent results in the fish oil and platelet aggregation literature. Furthermore, the majority of these hemostatic markers have been reported to be correlated or influenced by sex hormones [334, 463, 464] levels while sex hormone levels in turn influence LCn-3PUFA uptake and metabolism [196, 200]. Indeed we have previously demonstrated the existence of gender-dependant platelet responses with individual LCn-3PUFA both in vitro [441] and in vivo after short term exposures [312, 465]. The aim of the present investigation was thus to extend our findings to determine whether these gender-specific platelet responses are maintained with chronic LCn-3PUFA supplementation and further examine their effects on hemostatic markers. More specifically, we aimed to examine the gender-dependent effects of 4 week supplementation with EPA versus DHA rich oils on ex vivo platelet aggregation and plasma levels of TXB2 (the inactive metabolite of TXA2 which serves as a surrogate marker), P-sel, PAI-1 and vWF activity in healthy men and women.

5.1.3. Methods

5.1.3.1. Participants

This study was a randomised double-blinded, placebo-controlled intervention trial. A total of 94 healthy participants completed the study; 41 males and 53 females, recruited from the general community of Newcastle, NSW, Australia from June 2010 to May 2011. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m²; taking aspirin, anti-platelet medication or non-steroidal anti-inflammatory drugs. Participants were also excluded if they had previously consumed fish oil supplements or regularly consumed more than two seafood servings per week. Participants were asked to complete a medical questionnaire, 24 hour food recall and follow a diet low in tomatoes and seafood prior to the study day and during the intervention. All participants provided written informed consent according to governmental regulations concerning the ethical use
of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia (H-2010-0022) and has been registered in the Australian New Zealand Clinical Trials Registry (ACTRN1261100098932). The study was conducted in accordance with The Declaration of Helsinki. All work was conducted in the research unit at the Medical Sciences Building, University of Newcastle. All participants attended the research unit on 2 occasions. On the first visit participants completed a pre-trial medical history questionnaire and a 24 hour food record prior to a fasting blood sample donation.

Using computer generated methods; participants were block randomised with gender stratification to a treatment protocol of 2 x 1g capsules daily for 4 weeks of supplements containing either:

(i) Placebo (Sunola oil)
(ii) EPA rich oil supplement [500:100mg EPA/DHA (EPAX 5510 TG/N)]
(iii) DHA rich oil supplement [100:500mg EPA/DHA (EPAX 1050 TG/N)]

Participants and the co-investigators responsible for treatment allocation and assessment were blinded to the treatment groups. The supplements were masked and identifiable only by code to which only the principal study investigator had access to. Participants returned immediately after the intervention for a final fasting blood donation. Compliance to instructions and supplement intake was monitored via pre-intervention and post-intervention capsule count-back, telephone communication, analysis of plasma fatty acid composition and analysis of the daily record of capsule intake documented by participant.

5.1.3.2. Blood analysis
From each participant, venous blood was collected using a 21 gauge needle into vacutainer tubes containing 3.2% sodium citrate, EDTA, or lithium heparin following a > 10 hr fast. All samples were collected by the same venipucturist from the antecubital vein. Whole citrate blood was analyzed 20 min after collection for platelet aggregation assays. For measurement of TXB₂, P-sel, PAI-1 and plasma fatty acid composition, EDTA blood was centrifuged at 3000 x g for 10 min to obtain
platelet free plasma and aliquots were stored at -80°C until further analysis. For measurement of vWF activity, sodium citrate blood was centrifuged and stored as above. EDTA and lithium heparin tubes were used for analysis of full blood count and hormonal levels (testosterone, oestradiol), respectively, by Hunter New England Area Health Pathology Services (NSW, Australia) using standard analytical techniques.

5.1.3.3. Platelet aggregation assays
Platelet aggregation and lag time (time taken to initiate aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA) according to the method of Cardinal and Flower [411]. Whole blood (500 µL) was diluted with an equal volume of PBS. Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5µg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. Measurement variation was minimized by having the same trained technician process all samples using the same equipment.

5.1.3.4. Measurement of TXB2, vWF activity, P-sel and PAI-1
All plasma biomarkers were measured using commercially available immunoassay kits following the manufacturer’s instructions. Thromboxane B2 was quantitated using a competitive enzyme immunoassay (EIA) (Sapphire Bioscience, Waterloo, NSW, Australia), von Willebrand factor (vWF) activity was determined using a collagen binding assay (CBA) (Life Therapeutics, Clarkston, GA, USA), while soluble P-selectin and PAI-1 levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (R &D Systems, Minneapolis, MN, USA). All samples were analysed in duplicate and only those with a %CV < 15% were used in the final analysis. Replicate samples from individual participants were determined in the same series to avoid bias due to assay variability.

5.1.3.5. Plasma fatty acid analyses
The fatty acid composition of plasma lipids was determined according to a modification in the method of Lepage & Roy [417] using an acetyl chloride methylation procedure. Fatty acid methyl esters were quantified using GC (Hewlett Packard 6890; Hewlett Packard, Palo Alto, CA, USA). The identity of each fatty acid
peak was ascertained by comparison of the peaks’ retention time with those of synthetic standards of known fatty acid composition (Nu Check Prep, Elysian, MN, USA). The relative amount of each fatty acid was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids. Fatty acid results are reported as the percentage of total fatty acids.

5.1.3.6. Statistical analysis

Statistical analyses were performed using IBM Statistics software (version 20, SPSS Inc., Chicago). Sample size was calculated using means, standard deviation and power calculations from our previous data for comparison of platelet aggregation between males and females using a two-tailed t-test, an alpha of 0.05 and a power of 0.90. Based on this data the study was powered to detect a minimum 10% change in platelet aggregation and allowed for a 10% dropout rate due to unforeseen circumstances. All data are presented as mean ± SEM. Changes in hemostatic markers, platelet aggregation and plasma fatty acids were determined by calculating the difference from blood samples collected at 4 weeks post intervention with baseline values and expressed as a percentage change. Preliminary assumption testing using the Kolmogorov-Smirnov tests for normality, and stem and leaf plot showed that all data were normally distributed. Test for outliers and homogeneity of variance using showed that there were no serious violations observed for all test variables within sex and LCn-3PUFA group. Statistical significance was assessed using one-way ANOVA and the repeated measures t-test for paired data. Comparisons between gender and different LCn-3PUFA groups were made with general linear models using two way univariate and multivariate ANOVA with post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
5.1.4. Results

5.1.4.1. Baseline demographics

The average participant age was 39.6 ± 1.7 y without significant age differences between the male and female cohort. Approximately 70% of the female cohort was of premenopausal status and female participants in the study had significantly higher platelet count, higher levels of oestradiol, as well as significantly greater baseline platelet aggregation than males. Males had significantly greater BMI (kg/m^2), higher hematocrit, RBC (red blood cell) count, longer lag time (secs) and higher levels of testosterone and plasma EPA (Table 5-1). When assigned to each treatment group, there were no significant differences in these measures between the males in each treatment group or between the females in each group. No baseline differences were observed with subgroup analyses for sex within and between treatment groups for platelet aggregation biomarkers. EPA, n=31 (M:F = 13:18); DHA, n= 31 (M:F = 15:16); Placebo, n= 32 (M:F = 15:17). There were no losses to follow-up or exclusions through each phase of the study.
Table 5-1. Characteristics of study participants *

<table>
<thead>
<tr>
<th></th>
<th>Combined (n = 94)</th>
<th>Male (n = 43)</th>
<th>Female (n = 51)</th>
<th>P-value †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39.6 ± 1.7</td>
<td>40.8 ± 2.7</td>
<td>38.6 ± 2.2</td>
<td>0.523</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 0.4</td>
<td>25.6 ± 0.6</td>
<td>23.7 ± 0.5</td>
<td>0.013</td>
</tr>
<tr>
<td>Plt count (x 10⁹/L)</td>
<td>256 ± 6.1</td>
<td>237 ± 6.1</td>
<td>275 ± 6.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Hematocrit (L/L)</td>
<td>0.41 ± 0.003</td>
<td>0.49 ± 0.003</td>
<td>0.39 ± 0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RBC count (x 10¹²/L)</td>
<td>4.6 ± 0.04</td>
<td>4.8 ± 0.04</td>
<td>4.3 ±0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC count (x 10⁹/L)</td>
<td>5.6 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>0.091</td>
</tr>
<tr>
<td>Plt aggregation (AUC)</td>
<td>69.6 ± 1.2</td>
<td>66.2 ± 1.8</td>
<td>72.4 ± 1.7</td>
<td>0.012</td>
</tr>
<tr>
<td>Lag time (sec)</td>
<td>50.7 ± 0.02</td>
<td>56.4 ± 0.03</td>
<td>45.9 ± 0.02</td>
<td>0.012</td>
</tr>
<tr>
<td>TXB₂ (pg/mL )</td>
<td>122.3 ± 2.0</td>
<td>122.1 ± 3.2</td>
<td>122.5 ± 2.6</td>
<td>0.916</td>
</tr>
<tr>
<td>P-sel (ng/mL )</td>
<td>42.4 ± 1.8</td>
<td>41.3 ± 2.8</td>
<td>43.4 ± 2.3</td>
<td>0.560</td>
</tr>
<tr>
<td>vWF activity (%)</td>
<td>104.7 ± 5.9</td>
<td>99.2 ± 5.4</td>
<td>109.5 ± 10.2</td>
<td>0.376</td>
</tr>
<tr>
<td>PAI-1 (pg/mL )</td>
<td>25.3 ± 0.02</td>
<td>28.2 ± 0.04</td>
<td>22.9 ± 0.03</td>
<td>0.334</td>
</tr>
<tr>
<td>EPA (% of TFA)</td>
<td>1.15 ± 0.07</td>
<td>1.31 ± 0.13</td>
<td>1.01 ± 0.07</td>
<td>0.048</td>
</tr>
<tr>
<td>DHA (% of TFA)</td>
<td>2.71 ± 0.11</td>
<td>2.79 ± 0.17</td>
<td>2.65 ± 0.15</td>
<td>0.520</td>
</tr>
<tr>
<td>AA (% of TFA)</td>
<td>6.08 ± 0.29</td>
<td>5.95 ± 0.46</td>
<td>6.20 ± 0.40</td>
<td>0.680</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>8.4 ± 0.79</td>
<td>15.4 ± 0.62</td>
<td>1.44 ± 0.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oestradiol (pmol/L)</td>
<td>217.3 ± 35.4</td>
<td>50.1 ± 4.5</td>
<td>384.4 ± 56.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Values are mean ± SEM; † P-values were obtained using independent samples t-test for males vs. females. AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PAI-1, plasminogen activator inhibitor-1; P-sel, P-selectin; Plt, platelet; RBC, red blood cell; TFA, total fatty acid; TXB₂, thromboxane B₂; vWF, von Willebrand factor; WBC, white blood cell.
5.1.4.2. **Relationship between hormonal status, platelet aggregation, and plasma fatty acids prior to supplementation**

Significant correlations between sex and hemostatic variables were observed at baseline. Hematocrit and RBC count were positively associated with testosterone levels \( r = .637, P < 0.001 \) and \( r = .590, P < 0.001 \) respectively) while negatively associated with oestradiol levels \( r = -.396, P < 0.001 \) and \( r = -.319, P < 0.001 \) respectively. Baseline aggregation was positively associated with platelet count \( r = .452, P < 0.001 \) but inversely related to lag time \( r = -.489, P < 0.001 \) and testosterone levels \( r = -.215, P = 0.039 \). Accordingly, testosterone levels were negatively associated with platelet count \( r = -.302, P = 0.003 \) but positively associated with lag time \( r = 0.258, P < 0.05 \) indicating delayed time to aggregation.

The only plasma fatty acid to show any significant association was baseline plasma EPA, which was positively correlated with testosterone \( r = 0.222, P = 0.032 \) (Table 5-2).
### Table 5-2. Correlations between hormonal status and platelet aggregatory response*

<table>
<thead>
<tr>
<th></th>
<th>Baseline Plt aggregation (AUC)</th>
<th>Testosterone (nmol/L)</th>
<th>Oestradiol (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (L/L)</td>
<td>- .150</td>
<td>.637 †</td>
<td>- .396 †</td>
</tr>
<tr>
<td>RBC count (x 10^{12}/L)</td>
<td>-.158 †</td>
<td>.590 †</td>
<td>-.319 †</td>
</tr>
<tr>
<td>WBC count (x 10^{9}/L)</td>
<td>.305 †</td>
<td>-.124</td>
<td>.179</td>
</tr>
<tr>
<td>Platelet count (x 10^{9}/L)</td>
<td>.452 ‡</td>
<td>-.302 †</td>
<td>.189</td>
</tr>
<tr>
<td>Lag time (sec)</td>
<td>-.489 ‡</td>
<td>.258 †</td>
<td>.074</td>
</tr>
<tr>
<td>Baseline Plt aggregation (AUC)</td>
<td>-</td>
<td>-.215 †</td>
<td>.097</td>
</tr>
<tr>
<td>Baseline plasma EPA (% of TFA)</td>
<td>-.074</td>
<td>.222 †</td>
<td>-.114</td>
</tr>
<tr>
<td>Baseline plasma DHA (% of TFA)</td>
<td>-.152</td>
<td>.098</td>
<td>.003</td>
</tr>
<tr>
<td>Plt aggregation at 4 wks EPA (AUC)</td>
<td>-</td>
<td>-.379 †</td>
<td>.201</td>
</tr>
<tr>
<td>Plt aggregation at 4 wks DHA (AUC)</td>
<td>-</td>
<td>.430 †</td>
<td>-.251</td>
</tr>
</tbody>
</table>

*Values are bivariate correlation and Pearson coefficient; † Values indicate significance at $P<0.05$; ‡ Values indicate significance at $P\leq0.001$; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Plt, platelet; RBC, red blood cell; TFA, total fatty acid; WBC, white blood cell.

### 5.1.4.3. Effects of supplementation on platelet aggregation

When the entire cohort was considered, 4 weeks supplementation with either EPA or DHA rich oils effectively reduced platelet aggregation when compared to placebo by -11.8±2.7% ($P = 0.016$) and -14.8±2.2% ($P = 0.001$) respectively (Figure 5-1). As further depicted in figure 5-1, when stratified by gender, the aggregation response pattern was divided between the male and females subjects and a significant gender x treatment effect was observed ($P =0.001$). Furthermore, subgroup with post-hoc analyses showed that in males, only EPA effectively reduced platelet aggregation by -18.4±3.2% compared to a reduction of only -2.7±2.8% in the males taking placebo and -5.5 ±3.1% in females taking the same EPA supplement ($P = 0.011$). In contrast, in females only DHA decreased platelet aggregation with a -18.9±2.9%
overall reduction compared to only -2.1±2.9% in the females taking placebo ($P=0.001$) and -9.0±3.5% in the males consuming the same DHA supplement ($p=0.017$) (Figure 5-1).

These results were corroborated by analysis of associations with hormone levels: testosterone levels were inversely related to platelet aggregation activity after EPA supplementation ($r = -0.379$, $P = 0.035$), but positively associated after DHA supplementation ($r = 0.430$, $P = 0.016$) (Table 5-2). Furthermore, testosterone levels were inversely correlated with plasma DHA levels post DHA supplementation ($r = -0.37$, $P = 0.048$) (Table 5-2).

Table 5-3. Change (%) in hemostatic markers, platelet aggregation and plasma fatty acids post supplementation

<table>
<thead>
<tr>
<th></th>
<th>EPA</th>
<th>DHA</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔTXB₂ (pg/mL)</td>
<td>1.0 ± 1.3</td>
<td>1.1 ± 2.2</td>
<td>1.1 ± 1.9</td>
</tr>
<tr>
<td>ΔP-sel (ng/mL)</td>
<td>-3.3 ± 2.5</td>
<td>-4.4 ± 3.4</td>
<td>-0.8 ± 2.4</td>
</tr>
<tr>
<td>ΔvWF activity (%)</td>
<td>-5.1 ± 5.4</td>
<td>5.7 ± 4.9</td>
<td>-1.4 ± 5.8</td>
</tr>
<tr>
<td>ΔPAI-1 (ng/mL)</td>
<td>1.0 ± 3.4</td>
<td>1.9 ± 3.3</td>
<td>2.2 ± 3.4</td>
</tr>
<tr>
<td>ΔPlatelet aggregation (AUC)</td>
<td>-11.8 ± 2.7 †</td>
<td>-14.8 ± 2.2 ‡</td>
<td>-2.3 ± 2.0 †,‡</td>
</tr>
<tr>
<td>ΔPlasma EPA (% of TFA)</td>
<td>230.2 ± 28.3 †</td>
<td>151.0 ± 16.6 †</td>
<td>-3.2 ± 5.2 †</td>
</tr>
<tr>
<td>ΔPlasma DHA (% of TFA )</td>
<td>32.3 ± 7.5 †</td>
<td>117.5 ± 17.4 †,‡</td>
<td>-4.5 ± 5.5 §</td>
</tr>
<tr>
<td>ΔPlasma AA (% of TFA )</td>
<td>-7.6 ± 2.7</td>
<td>-6.6 ± 2.9</td>
<td>-3.1 ± 2.1</td>
</tr>
</tbody>
</table>

Results are mean % change ± SEM using one-way ANOVA with post hoc Tukey tests; †,‡ Values within a row sharing a common superscript are significantly different ($P<0.05$); AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Plt, platelet; TFA total fatty acid.
Table 5-4. Differential changes (%) in hemostatic markers, platelet aggregation and plasma fatty acids post supplementation in males and females *

<table>
<thead>
<tr>
<th></th>
<th>EPA</th>
<th></th>
<th>DHA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td></td>
<td>( n = 13)</td>
<td>( n = 18)</td>
<td>(n=15)</td>
<td>(n=16)</td>
</tr>
<tr>
<td>ΔTXB₂ (pg/mL)</td>
<td>-0.6 ± 1.4</td>
<td>2.8 ± 2.3</td>
<td>-1.6 ± 3.2</td>
<td>3.0 ± 2.9</td>
</tr>
<tr>
<td>ΔP-sel (ng/mL)</td>
<td>-3.2 ± 3.2</td>
<td>-3.3 ± 3.8</td>
<td>-2.7 ± 5.6</td>
<td>-5.3 ± 4.3</td>
</tr>
<tr>
<td>ΔvWF activity (%)</td>
<td>-9.4 ± 8.5</td>
<td>-1.1 ± 6.8</td>
<td>10.1 ± 5.6</td>
<td>2.3 ± 7.6</td>
</tr>
<tr>
<td>ΔPAI-1 (ng/mL)</td>
<td>-5.3 ± 2.0↑</td>
<td>1.9 ± 2.5↑</td>
<td>1.8 ± 5.1</td>
<td>7.3 ± 5.5</td>
</tr>
<tr>
<td>ΔPlatelet aggregation (AUC)</td>
<td>-18.4 ± 3.2↑,§</td>
<td>-5.5 ± 3.1↑,¶</td>
<td>-9.0 ± 3.5§,‡</td>
<td>-18.9 ± 2.9‡,¶</td>
</tr>
<tr>
<td>ΔPlasma EPA (% of TFA)</td>
<td>214.9 ± 41.6</td>
<td>244.6 ± 39.3</td>
<td>130.7 ± 20.9</td>
<td>165.2 ± 24.1</td>
</tr>
<tr>
<td>ΔPlasma DHA (% of TFA )</td>
<td>31.5 ± 13.3↑</td>
<td>33.0 ± 8.0↑,§</td>
<td>84.5 ± 18.7</td>
<td>148.3 ± 25.1</td>
</tr>
<tr>
<td>ΔPlasma AA (% of TFA )</td>
<td>-12.7 ±4.1</td>
<td>2.8 ± 3.2</td>
<td>-5.5 ± 3.8</td>
<td>-7.3 ± 4.3</td>
</tr>
</tbody>
</table>

* Results are mean % change ± SEM using independent samples t-test for males vs. females; ↑, §, †, ¶ Values within a row sharing a common superscript are significantly different (P<0.05); AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PAI-1, plasminogen activator inhibitor-1; P-sel, P-selectin; Plt, platelet; TXB₂, thromboxane B₂; TFA, total fatty acid; vWF, von Willebrand factor
5.1.4.4. **Markers of platelet activity and aggregation**

The percentage change in hemostatic markers, platelet aggregation and plasma fatty acids from baseline was examined for each group. When analysed by treatment group only, plasma TXB₂, vWF activity, P-sel and PAI-1 levels did not significantly change when compared to placebo (Table 6-3). When further stratified by gender, a significant effect was only observed in PAI-1 levels after supplementation with EPA (-5.3+2.0% for males vs. 1.9+2.5% for females, P = 0.046) (Table 5-4).

![Figure 5-1. Effect of treatment and gender on platelet aggregation following 4 weeks supplementation. Results are mean AUC % change ± SEM using two-way ANOVA with post hoc Tukey tests for LCn-3PUFA vs. placebo and for males vs. females where a significant gender x treatment effect was observed in the test of between-subject effects (P =0.001). Bars without a common symbol (*, †) are significantly different. P<0.05.](image-url)
5.1.4.5. Plasma fatty acid composition

Plasma fatty acid compositions were measured to ensure participant compliance in each treatment group. Plasma EPA levels were significantly increased in both EPA and DHA supplementation groups; by 230±28.3% (1.3±0.1 to 3.4±0.2 $P<0.001$) and 151±16.6% (0.9±0.1 to 2.2±0.1, $P<0.001$) respectively, but not in the placebo group (1.1±0.1 to 1.0±0.1, $P=0.175$). Similarly, plasma DHA levels were also significantly increased in both supplementation groups: by 117±17.4% with DHA supplementation (2.5±0.2 to 4.9±0.2, $P<0.001$) and 32±7.1% (2.7±0.1 to 3.3±0.1, $P<0.001$) with EPA supplementation, but not in the placebo group (2.9±0.2 to 2.6±0.1, $P=0.106$). In contrast, plasma AA levels were not significantly altered in any of the supplementation groups [-7.6±2.7% with EPA (6.1±0.5 to 5.8±0.5, $P=0.065$), -6.6±2.9% with DHA (5.8±0.5 to 5.4±0.4, $P=0.056$), and -3.1±2.1% (6.2±0.5 to 6.1±0.5, $P=0.415$) with placebo (Table 5-3).

Further exploration of treatment effects on plasma EPA and DHA between gender and within-treatment groups was undertaken. Using the MANOVA general linear model, a significant gender x treatment effect ($P=0.006$) was observed in the DHA supplementation group between males and females. While plasma EPA levels increased significantly to an equal extent in males and females supplemented with EPA (Figure 5-2A), plasma DHA levels were increased significantly more in females with 148%±25.1 compared to 84.5%±18.7 in males in the DHA supplementation group ($P=0.028$) (Figure 5-2B;Table 5-4). No significant changes in plasma EPA, DHA or AA were observed in the placebo group between or within treatment groups.
Figure 5-2. Comparative changes in plasma fatty acids following EPA (A) and DHA (B) supplementation in males and females. Results are mean plasma fatty acid composition % change ± SEM using two-way ANOVA with post hoc Tukey tests for treatment and sex. Bars without a common symbol (*, †, ‡) are significantly different. P<0.05. 

**Males** □ **Females**
5.1.5. Discussion

Several uncontrolled studies have reported conflicting results against fish oil supplementation on platelet aggregation [280-285] thus its putative effects remain unclear and controversial. We report for the first time in a controlled study, that the anti-aggregatory effects of EPA and DHA are gender-specific. Here, we offer a plausible explanation whereby sex; a non-modifiable determinant of CVD, may likely explain the inconsistent results in the fish oil and platelet aggregation literature.

In the present study, we have shown that gender-specific responses exist in platelet aggregation following four weeks of dietary supplementation with EPA or DHA rich oil capsules. These data confirm our previous findings of sex-dependant platelet responses in vitro [441] and ex vivo in an acute supplementation study [312, 466]; subsequently extending our finding that these gender-dependant anti-aggregatory effects of Lcn-3PUFA are maintained over longer terms. Consistent with our earlier findings, EPA-rich or DHA-rich oils were equally effective in reducing platelet aggregation following a four week supplementation period when the entire cohort was considered. Within gender sub-group analysis, we demonstrated a significant interaction between gender and treatment; the anti-aggregatory effects of EPA and DHA were clearly segregated into the male and female groups. It was evident that the effects of EPA were specific in reducing platelet aggregation in males with a marked -18.4% reduction whereas no effects were observed in the female cohort. Conversely, the effects of DHA were unique to females to a similar -18.9% decrease in platelet aggregability and without effect in males. This may explain the lack of effect of DHA supplementation on platelet aggregation in previously published studies conducted predominantly or exclusively in males [208, 280, 282]. Administration of testosterone has been shown to decrease plasma DHA levels [337] and to increase platelet TXA₂ receptor density and aggregation responses in healthy males [333]. The lack of effect observed in males with DHA supplementation in our study may be owing to the sex differences in Lcn-3PUFA metabolism whereby males synthesise and/or retain less DHA in comparison to females. Gender differences in Lcn-3PUFA metabolism have been well established; women during the child-bearing age have been shown to have higher concentrations of plasma DHA and lower concentrations of EPA compared with men and the difference is independent of dietary intake [351]. This was further supported by
Giltay et al where an increase in DHA status was observed with estrogen administration while in contrast, decreased with testosterone [337].

This in line with our findings where plasma DHA levels increased to a greater extent in females than males (148% vs. 64% respectively) following supplementation with DHA-rich capsules demonstrating a significant gender x treatment interaction ($P=0.006$). Considering that plasma fatty acid composition following chronic LCn-3PUFA supplementation reflects that of platelets [344], our data is consistent with the previously published in vitro study showing slower movement of DHA into platelet phospholipids in males [350]. Furthermore, testosterone levels were inversely correlated with plasma DHA levels post DHA supplementation. This is also consistent with the previous observations that testosterone administration reduce DHA levels [337] and increase platelet aggregation responses in healthy males [333]. This suggests that higher testosterone levels may regulate the retention (reduced uptake and/or increased oxidation) of DHA from the supplement, resulting in only limited inhibition of platelet aggregation in the males within our study. In further support of this, we observed a positive relationship between platelet aggregation at 4 weeks following DHA supplementation with testosterone levels. In contrast, higher levels of testosterone were associated with lower platelet aggregation activity following EPA supplementation. Similar to reported studies of 4-6 weeks supplementation [208, 356, 467], TXB$_2$ levels in our study subjects were not altered by EPA or DHA supplementation. It is likely that a longer supplementation period or higher doses are required to alter eicosanoid production. EPA and/or DHA supplementation is known to partially replace AA from the platelet phospholipids to compete as a substrate for the cyclooxygenase (COX) enzyme resulting in reduced conversion to TXA$_2$. TXA$_3$ derived from EPA is biologically less active compared to TXA$_2$ thereby reducing platelet activity [230].

Nonetheless, platelet aggregation was significantly reduced regardless of TXB$_2$ levels in our study. Coincidently no changes in plasma AA levels were evident while the EPA or DHA levels increased following dietary supplementation confirming both compliance to supplementation and no change in n-6 fatty acid dietary intake. It is likely that mechanisms other than eicosanoid production are responsible for the observed changes in platelet aggregation. Previous studies also reported no change in
TXA₂ levels, yet reduced platelet aggregation even with high doses of DHA (6g/day) [294] or EPA (10-40mL/day) [292]. Alternative mechanisms of action have been proposed including effects on platelet adhesion and platelet/endothelial cell activation [468]. P-sel and vWF are glycoproteins expressed on activated platelets. P-sel supports the recruitment of leukocytes and platelet adhesion [117] while vWF mediates platelet aggregation by binding platelets to collagen in the exposed endothelium [469], their inhibition has been shown to enhance thrombus dissolution [459]. In the present study, EPA rich oil supplementation in males decreased P-sel levels compared to DHA and placebo. Conversely, DHA supplementation induced similar effects in the females in comparison to EPA, placebo and the male group. Similar results were observed for vWF activity in males consuming EPA with considerably greater reduction when compared with DHA, placebo and the female group. Though these results did not reach significance, this trend is consistent with our present and previous findings suggesting that males may benefit more from EPA, while females are more responsive to DHA [441]. Additionally, a study by Eschen et al. reported that the effects of LC-n3PUFA on cellular adhesion molecules may also be dependent on gender and LCn-3PUFA dose [470].

Fibrinolysis, the final stage in hemostasis has been considered a target treatment approach to enhance dissolution of the thrombus commonly through their effects on plasminogen [471]. The degradation or the inhibition of fibrinolysis is tightly regulated by the interface between the plasminogen activators (PA) and plasminogen activator inhibitors (PAI) respectively [128]. The PAI-1 enzyme is the dominant PAI in the circulation, representing 60% of plasminogen activation inhibition in plasma. Subsequently, high levels of PAI-1 lead to a reduction in plasmin degradation of fibrin promoting thrombosis [472]. Conflicting results have been reported on the influence of fish oil on PAI-1 levels, some reporting impaired fibrinolytic capacity [313] while others find improvements [391] or without effects [407]. Interestingly, we observed a significant difference in PAI-1 levels between the male and female groups following EPA supplementation. PAI-1 levels decreased by a modest -5.3% in males while in females increased 1.9%. Environmental and genetic influences have been proposed with polymorphism of the PAI-1 gene, in particular the 4G allele where correlations between PAI-1 and triglyceride levels have been reported [473]. In addition, a large intra and interpersonal variability in plasma PAI-1 activity
coupled with diurnal variation has been reported [474] along with the influence of sex hormones upon PAI-1 synthesis [475, 476]. Although our study was not powered adequately, the changes in biomarkers of platelet adhesion and fibrinolysis were consistent with the gender-specific trend and collectively prompt further investigations. These observations also suggest that changes in ex vivo platelet aggregation do not necessarily correspond to changes in variables of primary hemostasis (platelet activation, adhesion). The gender-specific inhibitory effects on platelet aggregation observed with EPA and DHA could be via coagulation factors of secondary hemostasis which we anticipate to explore further.

In a recent study, the association between the baseline values of platelet aggregation, sex and hematological parameters were reported [477] which was consistent with our findings. Others have also reported that sex hormonal level is the confounding variable for these platelet differences observed [438, 478]. Furthermore, it is of interest to note that sex-specific effects have been reported with the anti-platelet action of aspirin use where it does not appear to be beneficial to women compared to men [479]. In the Nurse’s Health Study cohort, when stratified by aspirin use, LCn-3PUFA intakes were inversely associated with risk of thrombotic infarction, primarily among women who were not regular aspirin users [270]. A comparison between these studies and that reported here would be helpful in advancing the understanding of the sex-specific effects observed for thrombotic disease risk.

In the present study, we have demonstrated a significant gender x treatment interaction on platelet aggregation, PAI-1 levels and on plasma LCn-3PUFA uptake and furthermore established gender-specific associations between sex hormonal levels and platelet aggregation with their respective treatments. In attempts to resolve the existing controversy, our findings suggest that the effects of EPA and DHA on platelet aggregation are apparent; their effects are not shared or complementary, rather gender-specific. We have shown that these gender-specific, anti-thrombotic effects are extended to the fibrinolytic system with the reduction of PAI-1 levels accordingly. The significance of these findings provides insight into the intricate mechanism whereby EPA and DHA exert their effects. In addition, applicably; these novel findings allows for optimal cardio-protection tailored for both gender groups offering a safe and non-pharmacological approach. With respect to
thrombotic disease risk, men would likely benefit more from supplementation with EPA while women are more responsive to DHA.

5.2. Objective 5

5.2.1. Abstract
Long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA) comprised of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) offer cardio-protection involving a decrease in coagulant activity; however the evidence is equivocal. We have previously demonstrated that the acute (24h) effects of LCn-3PUFA supplementation on platelet aggregation in human subjects are gender-specific. This study investigated the mechanisms of the gender-dependent effects of LCn-3PUFA with 4 weeks supplementation of EPA versus DHA-rich oils on procoagulant and platelet activity in healthy subjects. A double-blinded placebo randomised controlled trial was conducted in 94 healthy adults: male (n=41) and female (n=53). Platelet aggregation and coagulation parameters including factors I, II, V, VII, VIII, IX, X, vWF:Ag and endogenous thrombin potential were measured at baseline and 4 weeks post-supplementation with EPA or DHA-rich oil capsules. We have previously reported that platelet aggregation is specifically reduced by supplementation with EPA in males, and DHA in females. This gender-specific effect was also observed for decreases in plasma levels of Factor II (-7.9 ± 3.8%, P =0.026), Factor V (-6.5 ± 4.5%, P=0.022) and vWF:Ag (-7.3±2.1%, P=0.034), and was most pronounced in males supplemented with EPA. In contrast, DHA mediated reduction in platelet aggregation in females was not accompanied by any significant changes in the coagulation parameters tested. Significant interactions between gender and specific LCn-3PUFA exist to reduce procoagulant activity differentially in males versus females and could have profound effects on managing risk of thrombotic disease.
5.2.2. Introduction

Consumption of LCn-3PUFA, comprised of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) is associated with reduction in incidence and severity of thrombotic diseases such as cardiovascular disease and stroke [258, 261]. But despite over three decades of study, the precise mechanisms of these effects remain to be elucidated. The effects of LCn-3PUFA on platelets and blood coagulation are variable [128]. Some studies suggest that LCn-3PUFA acts as an anti-thrombotic agent since it inhibits \textit{ex vivo} platelet aggregation activity [276, 275, 280, 480]. Conversely, others have reported that EPA/DHA have shown minimal benefit [282, 285]. Furthermore, some studies report a moderate reduction in Factor I (fibrinogen) levels, thrombin generation [359] and coagulation factors V, VII, and X [284, 363, 380, 381] with fish oil supplementation, whereas other groups fail to detect this [128, 166, 285]. In attempts to resolve this long standing conflict, the appreciation of independent effects of EPA and DHA on cardiovascular risk factors is now an emerging entity. Controlled human studies have demonstrated that DHA offers equally important cardio-protective effects, although it is often present in lower amounts in fish oil supplements [133, 481]. However, these studies controlling for dosage and concentration ratios of EPA:DHA alone, provided no further clarifications with respect to platelet function and hemostatic variables [133, 481].

This disparity concerning the effects of LCn-3PUFA on hemostatic parameters may be explained by gender-specific differences in platelet reactivity with EPA or DHA supplementation. The majority of studies, including the early research that laid the foundation for the cardio-protective effects of LCn-3PUFA, have ignored gender as a confounding factor and were conducted predominately in male subjects [212, 239, 275, 359]. We have previously demonstrated the existence of gender-specific platelet responses with individual LCn-3PUFA both \textit{in vitro} [441] and \textit{in vivo} after short term exposures [312, 482]. The aim of the present investigation was to extend our findings with long term LCn-3PUFA supplementation and examine their effects on procoagulant activity in parallel with \textit{ex vivo} platelet aggregation. More specifically, this study aimed to examine the mechanisms of the gender-dependent effects of LCn-3PUFA with 4 weeks supplementation of EPA versus DHA rich oils on procoagulant activity, thrombin generation, coagulation and platelet aggregation in healthy males and females.
5.2.3. Methods

5.2.3.1. Participants

This study was a double blinded, placebo controlled intervention trial. A total of 94 healthy participants completed the study; 41 males and 53 females, recruited from the general community of Newcastle, NSW, Australia from June 2010 to May 2011. Exclusion criteria were: diagnosed non-insulin dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m^2; taking aspirin, anti-platelet medication or non-steroidal anti-inflammatory drugs. Participants were also excluded if they had consumed fish oil supplements or consumed more than two seafood servings per week. Participants were asked to complete a medical questionnaire, 24 hour food recall and follow a diet low in tomatoes and seafood prior to the study day and during the intervention. All participants provided written informed consent according to governmental regulations concerning the ethical use of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia (H-2010-0022) and has been registered in the Australian New Zealand Clinical Trials Registry (ACTRN1261100098932). The study was conducted in accordance with The Declaration of Helsinki. The research was conducted at the Medical Sciences Building, University of Newcastle: Callaghan Campus, and the Hunter Haematology Research Group, Calvary Mater Newcastle Hospital. All participants attended the university research unit on 2 occasions. On the first visit participants completed a pre-trial medical history questionnaire and a 24 hour food record prior to a fasting blood sample donation.

Participants were randomised using a computer-based random number generator with gender stratification to a treatment protocol of 2 x 1g capsules daily for 4 weeks of supplements containing either:

(i) Placebo (Sunola oil)
(ii) EPA rich oil supplement [500:100mg EPA/DHA (EPAX 5510 TG/N)]
(iii) DHA rich oil supplement [100:500mg EPA/DHA (EPAX 1050 TG/N)]
Participants and the co-investigators responsible for treatment allocation and assessment were blinded to the treatment groups. The supplements were masked and identifiable only by code to which only the principle study investigator had access. Participants returned immediately after the intervention for a final fasting blood donation. Compliance to instructions and supplement intake was monitored via pre-intervention and post-intervention capsule count-back, telephone communication, analysis of plasma fatty acid composition and analysis of the daily record of capsule intake documented by participant.

5.2.3.2. Blood analysis

Venous blood was collected into BD vacutainers (Becton Dickinson Biosciences, Ltd, NSW AUS) following a > 10 hr fast. Whole blood in 3.2% sodium citrate was analyzed 20 min after blood collection for platelet aggregation assays. For measurement of coagulation factors and thrombin generation, citrate blood was centrifuged at 1,500 x g for 15 min then 13,000 x g for 2 min to obtain platelet free plasma and aliquots were stored at -80°C until further analysis. For quantification of plasma fatty acid composition, blood was collected into EDTA tubes centrifuged at 3000 x g for 10 min and stored as above. Fasting blood was collected into EDTA and lithium heparin tubes for full blood count and analysis of hormonal levels (testosterone, oestradiol), respectively. These samples were analyzed by Hunter New England Area Health Pathology Services (NSW, Australia) using standard analytical techniques.

5.2.3.3. Platelet aggregation assays

Platelet aggregation and lag time (time taken to initiate aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Halvertown PA) according to the method of Cardinal and Flower [411]. Briefly, whole blood (500 µL) was diluted with an equal volume of phosphate buffered saline (PBS). Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5µg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. Measurement variation was minimized by having the same trained technician process all samples using the same equipment.
5.2.3.4. Determination of coagulation factor levels

All samples were rapidly thawed in a water bath at 37 °C prior to analysis. Standard coagulometric methods were used to detect factors II, V, VII, VIII, IX and X levels (%) on a Behring Coagulation System analyser (BCS; Siemens Healthcare Diagnostics Pty, Ltd. VIC, AUS), and fibrinogen assays were performed on the Sysmex CA-1500 coagulation system analyser (Dade Behring Inc. Newark, DE) using kits and reagents purchased from Dade Behring. Briefly, each participant’s individual plasma sample was mixed with the respective coagulation factor deficient plasma and a recombinant thromboplastin (Innovin) or activated partial thromboplastin time (Actin FSL) reagent and the time for clot formation measured in seconds. The amount of factor activity in the system was then quantified from a standard curve of the corresponding coagulation factor deficient plasma and known concentrations of a normal plasma plotted against the clotting time. Results are expressed as levels or activities (% of norm).

Plasma von Willebrand factor (vWF) activity was determined using a collagen binding assay (CBA) (Life Therapeutics, Clarkston, GA, USA) following the manufacturer’s instructions. vWF antigen (vWF:Ag) was measured on a BCS according to standard immunoturbidmetric methods as per the manufacturer’s instructions. Briefly, a suspension of polystyrene particles coated with vWF-specific antibodies was mixed with each participant’s plasma, and agglutination was detected via increase in turbidity which was proportional to the antigen level present in the participant’s sample.

5.2.3.5. Endogenous Thrombin Potential (ETP)

Thrombin generation was performed at 37°C on a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Finland) and analysed using the Calibrated Automated Thrombogram (Thrombinscope BV, Maastricht, the Netherlands) according to the manufacturer’s instructions. Briefly, 80 μl of platelet-free plasma was diluted with 20 μl of PPP reagent (containing 5 pM recombinant tissue factor and 4 μM phospholipids), and 20 μl of FluCa solution (Hepes, pH 7.35, 100 nM CaCl₂, 60 mg/mL bovine albumin, and 2.5 mM Z-Gly-Gly-Arg-amido methyl coumarin). The mean AUC corresponding to the ETP (nM x min) of duplicate tests was used for analysis. The intra-assay variability was calculated to be 1.7%.
5.2.3.6. **Plasma fatty acid analyses**

The fatty acid composition of plasma lipids was determined according to a modified method [417] where an acetyl chloride methylation procedure was appropriated. Fatty acid methyl esters were quantified using gas chromatography GC (Hewlett Packard 6890; Hewlett Packard, Palo Alto, CA, USA). The identity of each fatty acid peak was ascertained by comparison of the peak’s retention time with the retention times of synthetic standards of known fatty acid composition (Nu Check Prep, Elysian, MN, USA). The relative amount of each fatty acid was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids. Fatty acid results are reported as percentage of total fatty acids.

5.2.3.7. **Statistical analysis**

Statistical analyses were performed using IBM Statistics software (version 20, SPSS Inc., Chicago). Sample size was calculated using means, standard deviation and power calculations from our previous data for comparison of platelet aggregation between males and females using a two-tailed t-test, an alpha of 0.05 and a power of 0.90. Based on this data the study was powered to detect a minimum 10 % change in platelet aggregation. All data are presented as mean ± SEM. Changes in coagulation factors, platelet aggregation and plasma fatty acids were determined by calculating the difference from blood samples collected at 4 weeks post intervention with baseline values and expressed as a percentage change. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test variables within gender and LCn-3PUFA group. Statistical significance was assessed using one way ANOVA and the repeated measures t-test for paired data. Comparisons between gender groups and different LCn-3PUFA groups were made with general linear models using two way univariate and multivariate ANOVA with post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.
5.2.4. Results

5.2.4.1. Baseline demographics

The average age of the cohort was 39.6 ± 1.7 y, and the BMI was 24.6 ± 0.3 kg/m² (Table 5-5). All factor concentrations were within the normal range [483]. The female participants had significantly higher platelet count, platelet aggregability and higher plasma levels of FV and oestradiol than the males. Conversely, the males had significantly greater BMI, higher levels of testosterone as well as plasma EPA. There were no significant differences between the males and the females in each treatment group [EPA, n=31 (M:F = 13:18); DHA, n=31 (M:F = 15:16); Placebo, n=32 (M:F = 15:17)] (data not shown).
Table 5-5. Characteristics of study participants *

<table>
<thead>
<tr>
<th></th>
<th>Combined</th>
<th>Male</th>
<th>Female</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 94)</td>
<td>(n = 43)</td>
<td>(n = 51)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>39.6 ± 1.7</td>
<td>40.8 ± 2.7</td>
<td>38.6 ± 2.2</td>
<td>0.523</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 0.4</td>
<td>25.6 ± 0.6</td>
<td>23.7 ± 0.5</td>
<td>0.013</td>
</tr>
<tr>
<td>Plt count (x 10⁹/L)</td>
<td>256 ± 6.1</td>
<td>237 ± 6.1</td>
<td>275 ± 6.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Plt aggregation (AUC)</td>
<td>69.6 ± 1.2</td>
<td>66.2 ± 1.8</td>
<td>72.4 ± 1.7</td>
<td>0.012</td>
</tr>
<tr>
<td>Prothrombin (%)</td>
<td>107.9 ± 2.6</td>
<td>122.1 ± 3.2</td>
<td>122.5 ± 2.6</td>
<td>0.886</td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>103.3 ± 2.5</td>
<td>103.5 ± 3.6</td>
<td>103.1 ± 3.5</td>
<td>0.925</td>
</tr>
<tr>
<td>Factor VII (%)</td>
<td>110.3 ± 2.9</td>
<td>107.4 ± 3.9</td>
<td>112.7 ± 4.1</td>
<td>0.364</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
<td>119.8 ± 3.8</td>
<td>116.1 ± 5.7</td>
<td>123.2 ± 5.0</td>
<td>0.347</td>
</tr>
<tr>
<td>Factor IX (%)</td>
<td>97.7 ± 1.6</td>
<td>95.3 ± 2.1</td>
<td>94.2 ± 2.4</td>
<td>0.749</td>
</tr>
<tr>
<td>Factor X (%)</td>
<td>115.3 ± 2.0</td>
<td>109.9 ± 2.6</td>
<td>119.8 ± 2.9</td>
<td>0.014</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.8 ± 0.06</td>
<td>2.6 ± 0.09</td>
<td>2.9 ± 0.08</td>
<td>0.057</td>
</tr>
<tr>
<td>vWF antigen (U/dL)</td>
<td>109.9 ± 3.8</td>
<td>107.9 ± 4.9</td>
<td>111.7 ± 5.7</td>
<td>0.623</td>
</tr>
<tr>
<td>vWF activity (%)</td>
<td>104.7 ± 5.9</td>
<td>99.2 ± 5.4</td>
<td>109.5 ± 10.2</td>
<td>0.376</td>
</tr>
<tr>
<td>ETP (nM x min)†</td>
<td>17.2 (16.7-17.7)</td>
<td>16.8 (16.1-17.4)</td>
<td>17.5 (16.8-18.3)</td>
<td>0.137</td>
</tr>
<tr>
<td>EPA (% of TFA)</td>
<td>1.15 ± 0.07</td>
<td>1.31 ± 0.13</td>
<td>1.01 ± 0.07</td>
<td>0.048</td>
</tr>
<tr>
<td>DHA (% of TFA)</td>
<td>2.71 ± 0.11</td>
<td>2.79 ± 0.17</td>
<td>2.65 ± 0.15</td>
<td>0.520</td>
</tr>
<tr>
<td>AA (% of TFA)</td>
<td>6.08 ± 0.29</td>
<td>5.95 ± 0.46</td>
<td>6.20 ± 0.40</td>
<td>0.680</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>8.4 ± 0.79</td>
<td>15.4 ± 0.62</td>
<td>1.44 ± 0.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oestradiol (pmol/L)</td>
<td>217.3 ± 30.5</td>
<td>50.1 ± 4.5</td>
<td>384.4 ± 56.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Values are mean ± SEM ; † P-values were obtained using independent samples t-test for males vs. females. ; ‡ Values reported are x10²; lower and upper quartiles are reported in parentheses; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETP, endogenous thrombin potential; Plt, platelet; vWF, von Willebrand factor.
5.2.4.2. **Overall effects of supplementation**

Analysis by treatment group showed that supplementation with EPA or DHA rich oils for 4 weeks effectively reduced platelet aggregation compared to placebo by -11.8±2.7% (P = 0.016) and -14.8±2.2% (P = 0.001) respectively (Figure 5-3A, Table 5-6). However, there was no significant change in any of the hemostatic parameters examined. Plasma fatty acid measurements indicated that all participants were compliant in their treatment. In the EPA supplementation group, both the EPA and DHA levels were significantly increased by 230±28.3% (1.3±0.1 to 3.4±0.2 % of TFA; P<0.001) and 32±7.1% (2.7±0.1 to 3.3±0.1 % of TFA, P<0.001) respectively. Similarly, in the DHA supplementation group both the EPA and DHA were significantly increased by 151 ±16.6% (0.9±0.1 to 2.2±0.1 % of TFA, P<0.001) and 117±17.4% (2.5±0.2 to 4.9±0.2 % of TFA, P<0.001) respectively (Table 2). Accordingly, in the placebo group neither the EPA nor DHA levels were significantly increased [-3.2±5.2% (1.1±0.1 to 1.0±0.1 % of TFA, P=0.175) and -4.5%±5.5% (2.9±0.2 to 2.6±0.1 % of TFA, P=0.106) respectively]. Plasma arachidonic acid (AA) levels were also not significantly different in any of the supplementation groups (Table 5-6).
Figure 5-3. Differential effects of supplementation on hemostatic variables and platelet aggregation in males and females combined (A), male cohort (B) and female cohort (C). Results are mean % change using two-way ANOVA with post hoc Tukey tests for LCn-3PUFA vs. placebo and for males vs. females where a significant gender x treatment effect was observed in the test of between-subject effects ($P < 0.05$).
Table 5-6. Change (%) in coagulation profile, hemostatic parameters and plasma fatty acids following dietary supplementation with EPA or DHA rich oils*

<table>
<thead>
<tr>
<th></th>
<th>EPA (n = 31)</th>
<th>DHA (n = 31)</th>
<th>Placebo (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆Plt aggregation (AUC)</td>
<td>-11.8 ± 2.7†</td>
<td>-14.8 ± 2.2†</td>
<td>-2.3 ± 2.0*§</td>
</tr>
<tr>
<td>∆Prothrombin (%)</td>
<td>-3.1 ± 3.0</td>
<td>0.9 ± 1.9</td>
<td>1.5 ± 1.7</td>
</tr>
<tr>
<td>∆Factor V (%)</td>
<td>-2.1 ± 3.4</td>
<td>-2.2 ± 2.9</td>
<td>2.5 ± 2.8</td>
</tr>
<tr>
<td>∆Factor VII (%)</td>
<td>-6.1 ± 2.9</td>
<td>3.6 ± 2.9</td>
<td>-1.5 ± 3.0</td>
</tr>
<tr>
<td>∆Factor VIII (%)</td>
<td>-1.5 ± 2.2</td>
<td>-0.8 ± 3.4</td>
<td>-1.7 ± 2.1</td>
</tr>
<tr>
<td>∆Factor IX (%)</td>
<td>-2.4 ± 1.4</td>
<td>-2.2 ± 1.5</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>∆Factor X (%)</td>
<td>-1.6 ± 1.9</td>
<td>0.7 ± 1.8</td>
<td>-1.7 ± 2.0</td>
</tr>
<tr>
<td>∆Fibrinogen (g/L)</td>
<td>-2.1 ± 1.7</td>
<td>-1.1 ± 1.8</td>
<td>2.0 ± 3.2</td>
</tr>
<tr>
<td>∆vWF antigen (U/dL)</td>
<td>-4.3 ± 1.6</td>
<td>-0.1 ± 3.4</td>
<td>0.2 ± 1.8</td>
</tr>
<tr>
<td>∆vWF activity (%)</td>
<td>-5.1 ± 5.4</td>
<td>5.7 ± 4.9</td>
<td>-1.4 ± 5.8</td>
</tr>
<tr>
<td>∆ETP (nM x min)</td>
<td>-1.4 ± 1.5</td>
<td>0.8 ± 1.3</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>∆EPA (% of TFA)</td>
<td>230.2 ± 28.3†*</td>
<td>151.0 ± 16.6†§</td>
<td>-3.2 ± 5.2*§</td>
</tr>
<tr>
<td>∆DHA (% of TFA)</td>
<td>32.3 ± 7.5*</td>
<td>117.5 ± 17.4†§</td>
<td>-4.5 ± 5.5*</td>
</tr>
<tr>
<td>∆AA (% of TFA)</td>
<td>-7.6 ± 2.7</td>
<td>-6.6 ± 2.9</td>
<td>-3.1 ± 2.1</td>
</tr>
</tbody>
</table>

* Results are mean % change ± SEM using one-way ANOVA with post hoc Tukey tests; † Values are significantly different compared to placebo (P<0.05); § Values are significantly different compared to DHA (P<0.05); AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETP, endogenous thrombin potential; Plt, platelet; vWF, von Willebrand factor.
5.2.4.3. Gender specific effects of supplementation

When stratified by gender, a significant gender x treatment effect was observed ($P = 0.001$; Table 5-7). Further subgroup analyses showed that in males, only the EPA effectively reduced platelet aggregation (-18.4±3.2%), when compared to the placebo, (-2.7±2.8%, $P = 0.005$; Figure 5-3B), and to the female group (-5.5 ±3.1%, $P = 0.011$; Table 5-7). Conversely, in the female group, only DHA significantly reduced platelet aggregation (-18.9±2.9%) compared to the placebo (-2.1±2.9, $P=0.001$; Figure 5-3C) and the male group (-9.0±3.5, $P = 0.017$; Table 5-7). This gender specific effect was also observed for changes in prothrombin ($P =0.026$), Factor V activity ($P=0.022$) and vWF:Ag measurements ($P=0.034$; Table 5-7), however this phenomenon was more evident in males who were supplemented with EPA. In this subgroup, prothrombin levels were significantly reduced by -7.9±3.8%, as compared to the placebo, 5.5±2.0% ($P = 0.005$), 3.0 ±1.9% ($P = 0.039$) when compared to males supplemented with DHA only. Factor V levels were also significantly reduced exclusively in this group when compared to the placebo group (-6.5±4.5% vs. 10.2±3.9% respectively, $P=0.027$) (refer to Figure 5-3B).

A decrease in the vWF:Ag levels in this same EPA supplemented male group was observed between treatment groups ($P=0.034$), this (-7.3±2.1%) did not reach significance, when compared between treatment groups ($P=0.059$). Supplementation with EPA did not significantly affect any of the other coagulation parameters tested. In addition, no effects were observed with DHA supplementation between treatment groups or within gender subgroups. In contrast to the coagulation profiles, plasma fatty acid compositions indicated the existence of a significant gender x treatment effect with DHA treatment ($P=0.006$), where plasma DHA levels were increased significantly more in females compared to males (148%±25.1 compared to 84.5%±18.7 respectively; $P=0.028$).

5.2.4.4. Relationship between hormonal status, platelet aggregation, procoagulant activity and plasma fatty acid composition in response to supplementation

Confirming the associations with gender described above, testosterone levels were inversely related to changes in platelet aggregation post EPA supplementation ($r= -0.379$, $P= 0.035$; Table 5-8) but positively associated to changes in platelet
aggregation post DHA supplementation ($r = 0.430, P = 0.016$). Furthermore, lower oestradiol levels were associated with the observed decrease in prothrombin and FV activity following EPA supplementation ($r = 0.379, P = 0.036$; $r = 0.449, P = 0.01$ respectively). The reduction in platelet aggregation post EPA supplementation was best correlated with a decrease in vWF levels ($r = 0.361, P = 0.046$).
Table 5-7. Differential effects of EPA or DHA rich oil supplementation on coagulation profiles and plasma FAs in males and females*  

<table>
<thead>
<tr>
<th></th>
<th>EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males (n = 13)</td>
<td>Females (n = 18)</td>
</tr>
<tr>
<td>∆Plt aggregation (AUC)‖</td>
<td>-18.4 ± 3.2 †‡</td>
<td>-5.5 ± 3.1 †‡</td>
</tr>
<tr>
<td>∆Prothrombin (%)‖</td>
<td>-7.9 ± 3.8 †‡</td>
<td>1.3 ± 4.4</td>
</tr>
<tr>
<td>∆Factor V (%)‖</td>
<td>-6.8 ± 3.5 †</td>
<td>2.1 ± 5.1</td>
</tr>
<tr>
<td>∆Factor VII (%)</td>
<td>-7.3 ± 4.1</td>
<td>-4.9 ± 4.1</td>
</tr>
<tr>
<td>∆Factor VIII (%)</td>
<td>-3.6 ± 3.1</td>
<td>0.6 ± 3.1</td>
</tr>
<tr>
<td>∆Factor IX (%)</td>
<td>-3.8 ± 1.9</td>
<td>-1.1 ± 1.9</td>
</tr>
<tr>
<td>∆Factor X (%)</td>
<td>-0.5 ± 2.6</td>
<td>-2.6 ± 2.9</td>
</tr>
<tr>
<td>∆Fibrinogen (g/L)</td>
<td>-4.4 ± 2.5</td>
<td>0.1 ± 2.2</td>
</tr>
<tr>
<td>∆vWF antigen (U/dL)‖</td>
<td>-7.3 ± 2.1</td>
<td>-1.8 ± 2.2</td>
</tr>
<tr>
<td>∆vWF activity (%)</td>
<td>-9.4 ± 8.5</td>
<td>-1.1 ± 6.8</td>
</tr>
<tr>
<td>∆ETP (nM x min)</td>
<td>-2.8 ± 2.7</td>
<td>-0.1 ± 1.6</td>
</tr>
<tr>
<td>∆EPA (% of TFA)</td>
<td>214.9 ± 41.6 †‡</td>
<td>244.6 ± 39.3 †‡</td>
</tr>
<tr>
<td>∆DHA (% of TFA)‖</td>
<td>31.5 ± 13.3 †‡</td>
<td>33.0 ± 8.0 †‡</td>
</tr>
<tr>
<td>∆AA (% of TFA)</td>
<td>-12.7 ± 4.1</td>
<td>2.8 ± 3.2</td>
</tr>
</tbody>
</table>
Results are mean % change ± SEM; † Values are significantly different compared to placebo (P<0.05); ‡ Values are significantly different compared to DHA (P<0.05); § Values are significantly different compared to EPA (P<0.05); ‖ Significant gender x treatment effect (P<0.05); AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETP, endogenous thrombin potential; Plt, platelet; vWF, von Willebrand factor.

Table 5-8. Correlations between hormonal status, coagulation activity and platelet aggregatory response following 4 weeks EPA/DHA supplementation*

<table>
<thead>
<tr>
<th></th>
<th>Testosterone (nmol/L)</th>
<th>Oestradiol (pmol/L)</th>
<th>Platelet aggregation (AUC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF (U/dL)</td>
<td>-.114/.361†</td>
<td>.140/-0.043</td>
<td>.361†/.215</td>
</tr>
<tr>
<td>Prothrombin (%)</td>
<td>-.337/.204</td>
<td>.379/.113</td>
<td>.180/-0.295</td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>-.258/.237</td>
<td>.449/.167</td>
<td>.070/.169</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td>-.379†/.430†</td>
<td>.251/-0.201</td>
<td>-</td>
</tr>
</tbody>
</table>

* Values are bivariate correlation and Pearson coefficient of EPA/DHA respectively; † Values indicate significance at P<0.05; ‡ Values indicate significance at P≤0.01.

5.2.5. Discussion

With respect to the anti-aggregatory effects of LCn-3PUFA, we have previously shown that males are more responsive to acute EPA supplementation while female platelets respond primarily to DHA [312]. In the present study, we demonstrate that these gender-specific effects are maintained following chronic supplementation with LCn-3PUFA and in addition influence both primary (platelet function) and secondary (procoagulant activity) hemostasis.

LCn-3PUFA content in tissues is important in maintaining a balanced lipid profile as lipid abnormalities contribute substantially to the thrombotic process [340]. Incorporation of EPA/DHA in platelet membrane alters the phospholipid composition inducing subtle changes both in the inner and the outer plasma membrane. This is of significance given that the outer platelet membrane provides the catalytic surface for the binding of FV, cleavage of prothrombin and subsequent
adhesion and aggregation of platelets. Indeed in our study the substantial increases in plasma EPA and DHA with their respective treatments in both males and females demonstrated not only compliance to treatment but further provided evidence of gender differences in LCn-3PUFA uptake as reported in previous studies [339]. Plasma EPA levels were increased significantly with EPA-rich supplementation demonstrating efficient EPA uptake into membrane phospholipids [344]. This preferential incorporation of EPA into phospholipids 3-4 fold higher than DHA has been previously reported in platelets in a cohort of males [350]. The lack of effect observed in males with DHA supplementation in our study could be explained by gender differences in LCn-3PUFA uptake whereby males are unable to retain sufficient DHA in comparison to females [206]. Furthermore, administration of estrogen results in an increase in DHA status while in contrast is decreased with testosterone [337]. Similarly in our study, testosterone levels were inversely correlated with plasma DHA levels following DHA supplementation, while increased platelet aggregation activity following DHA supplementation was associated with higher testosterone levels. Our gender-dependent results regarding fatty acid uptake parallel the gender-specific decrease in procoagulant activity and collectively reflect the reported literature.

Reduced thrombotic tendencies can arise not only from altered platelet function but also from modifications in procoagulant activity; both being intimately linked and mutually stimulatory [484]. Similar to reports of different effects on platelet response, inconsistent results on the coagulation system using varying concentrations and/or ratios of fish oils in male or mixed gender cohorts have been reported [166, 280, 282, 359]. Notably, when our data was analysed by treatment group only, procoagulant activity was not altered with either EPA or DHA supplementation. However we were able to demonstrate the existence of gender and treatment interactions, whereby EPA supplementation in males specifically reduced prothrombin, Factor V and vWFAg levels, but no effect was seen with DHA supplementation. This validates studies reporting anti-coagulant effects of fish oils in all male cohorts where oils rich in EPA were used [290, 295, 359]. Moreover, this confirms reports where no changes in blood coagulation profiles or platelet aggregation were observed in healthy male subjects supplemented with 6g/day of DHA [282], or with EPA:DHA ratios ≤1:1 [280, 285, 403].
In contrast to other studies, we did not detect changes observed in Factor 1 (fibrinogen) and Factors VII and X, each of which is implicated in thrombin formation [215, 363, 380]. Additionally, thrombin generation was also not affected in our study cohort. Furthermore, thrombin generation is dependent on the initial prothrombin concentration [485] which in our study prothrombin (Factor II) was decreased with EPA supplementation. In one study the lowering effect of fish oil on thrombin generation and fibrinogen were reported to be clustered around subjects with high fibrinogen carrying a structural fibrinogen α-chain polymorphism [359]. In addition, fibrinogen and FX levels have shown to be lowered with fish oil in those with higher respective baseline levels [363, 381], and hypercoagulant profiles [380]. Thus a possible explanation for the minimal effect in fibrinogen or FX observed in the current study is that baseline concentrations were at the lower end of normal, and subjects with higher concentrations may be necessary to demonstrate a decline.

Nonetheless, prothrombin and FV activity were reduced with EPA supplementation in our male subjects. Factor V is an enzymatically inactive protein that serves as a cofactor for activated FX(a) to enable the assembly of the prothrombinase complex on the surface of activated platelets [84, 486]. Subsequently, prothrombin that is bound to glycoprotein GPIIb/IIIa complex within the platelet membrane is cleaved by FXa resulting in the release of thrombin [82]. The production of thrombin from prothrombin in turn stimulates platelet activation [70] which is mediated by adhesive proteins such as vWF to allow platelet interaction. The outcome is cross-linking of GPIIib/IIIa receptors on adjacent platelets and subsequent platelet aggregation [50]. Essentially, the adhesion of platelets to collagen mediated by vWF activates platelets for aggregation and proteins for coagulation. Indeed, increases in prothrombin, FV and vWF serve as potential risk factors for thrombotic events. Elevated prothrombin and FV activity levels are associated with arterial and venous thrombosis [251, 487-490] and reduction of plasma vWF activity decreases risk in cardiac patients [491].

It is well established that sex hormones are potent modulators of cardiac risk factors during the disease state as they alter procoagulant protein expression and the function of blood and vascular cells [314, 315]. In addition, gender-specific tissue and cellular characteristics also mediate sex-specific responses to a variety of stimuli where differences in platelet GPIIib/IIIa activation have been described with varying sex
hormone concentrations [317]. Moreover, inverse relationships between serum testosterone and the degree of atherosclerosis have been reported [492, 493], thus it is speculated that hemostatic mechanistic responses and their subsequent modulation by diet and/or drugs may differ in accordance with varying sex hormonal concentrations.

Interestingly, interactions between female hormones with prothrombin and FV Leiden mutation genotype have been suggested. Mutational variations with prothrombin and Factor V are associated with female hormones where increased frequencies have been detected in women [494, 495]. It can be postulated that studies including females or not stratifying by gender may have resulted in variable effects which contributed to the inconsistency of fish oil intervention on the coagulant potential. Indeed, the observed decrease in prothrombin and FV activity following EPA supplementation in our cohort was associated with lower oestradiol levels ($r=0.449, P=0.01$). A limitation of this study is the lack of genotyping to associate with these variations.

The findings from our present study provide the first indication that gender contributes to differential platelet and procoagulant responses according to EPA or DHA supplementation. EPA-rich oils reduces platelet and procoagulant activity in healthy males while DHA-rich oils reduce platelet activity in females towards the direction of reduced thrombotic disease risk. More studies are required to clarify this aspect of LCN-3PUFA interactions with the hemostatic system. However, this gender-dependent regulation may have important implications for understanding the basis of the gender gap in thrombotic complications. Given the toll taken by thrombotic diseases, a mechanistic understanding of how the role of gender functions as a disease modifier would be highly desirable.
Chapter Six: General Discussion
6.1. Key findings

EPA and DHA present different effects on several functions in cells and these differences are associated with their effects on membrane physicochemical properties [496], eicosanoid production [225, 233] intracellular signalling pathways [497] and vasoreactivity [498]. The marked differences between the effects of EPA and DHA indicate that it is an over-simplification to generalize the effects of LCn-3PUFA on cell function. There is limited data directly comparing platelet variables with individual effects of EPA versus DHA in humans. To date, it is not known whether the anti-aggregatory effects are owing to EPA or DHA and whether differences exist in the efficacy of individual LCn-3 PUFA to reduce platelet aggregation differentially.

This thesis has examined the pathways whereby EPA and DHA influence the hemostatic response differentially with exploration of both platelet and procoagulant activity translating to hemostatic factors of primary and secondary hemostasis respectively. The published findings from study 1 (Chapter 3), demonstrated for the first time the existence of gender-specific responses in platelet aggregation following treatment with individual LCn-3PUFA in healthy males and females. Specifically, the study demonstrated the efficacy of treatment with individual LCn-3PUFA to inhibit in vitro platelet aggregation was equally effective in females, while only treatment with EPA was effective in males. This original research article has also been the first to demonstrate the functional effects of DPA on platelet aggregation in humans. The importance of these findings presented in this paper was recognised by the Nutrition Society of Australia and the Nestle Nutrition Research Institute. This recognition earned success as the recipient of the ‘Nestle Nutrition Emerging Researcher Award 2009’ as an original research paper with outstanding implications and potential contributions to nutrition and cardiovascular health.

The publications presented in Chapter 4 confirmed the in vitro findings and demonstrated for the first time in a human clinical trial, the existence of gender-specific responses in ex vivo platelet aggregation and platelet microparticle activity
after a single oral dose of omega-3 fatty acids in healthy males and females. Publication 4 demonstrated that efficacy of treatment with a single oral dose of the EPA rich oils to reduce platelet aggregation is effective in males only, while in contrast the effect of DHA is unique to females. Subsequently, the accompanying published manuscript, Publication 5 reported a reduction in platelet microparticle activity in parallel with the reduction in platelet aggregation following EPA supplementation in males. Consistent with this finding, microparticle procoagulant activity was positively associated with platelet aggregation in the male cohort only. The publications from this chapter were the first to demonstrate and monitor the functional effects of the varying concentration ratios of EPA versus DHA rich oils on platelet function in humans over a 24 hour period.

Having demonstrated these gender-biased platelet responses both in vitro (Study 1) and in an acute feeding trial (Study 2), the final study (Study 3) explored these effects with chronic supplementation over a 4 week intervention.

Chapter 5 reports study 3 and demonstrated that the gender-dependant platelet responses to LCn-3PUFA are apparent over longer term exposures of 4 weeks. In this chapter, Paper 6 reports significant gender x treatment interactions on platelet activity and plasma LCn-3PUFA uptake. We have shown that these gender-specific, anti-thrombotic effects are extended to the fibrinolytic system with the reduction of PAI-1 levels accordingly. Paper 7 extended these findings to reflect the gender-specific differences on procoagulant activity in the coagulation pathway. In males, chronic supplementation with EPA reduced platelet aggregation, prothrombin and factor V activity, while in females; platelet aggregation was reduced with DHA supplementation only. Confirming the associations with gender described above, testosterone levels were inversely related to changes in platelet aggregation post EPA but positively associated to changes in platelet aggregation post DHA supplementation. Furthermore, lower oestradiol levels were associated with the observed decrease in prothrombin and FV activity following EPA supplementation and the reduction in platelet aggregation post EPA supplementation correlated with a decrease in vWF levels.
Collectively, the findings from these series of research studies incorporating in vitro, acute and chronic dietary LCn-3PUFA supplementation trials provide a plausible explanation for the variable results in the literature in the anti-aggregatory and anticoagulant response with fish oil supplementation. In males, EPA-rich oil supplementation attenuated platelet aggregation via extension of lag time (time taken to initiate aggregation). The increased lag time demonstrated in these studies suggest that EPA may reduce aggregation through weakened or delayed responses to aggregatory agents thereby increasing prothrombin time and bleeding time [295]. Pharmacokinetic data demonstrated that lag time progressively increased with EPA supplementation in males in accordance with the continuous decrease in platelet aggregation over 24 hours (Chapter 4). This has significant applications given that lag time is indicative of platelet responsiveness where an association between platelet responsiveness and cardiovascular deaths has been reported in a study involving healthy males [446]. Males with enhanced platelet reactivity characterised by shorter lag time, had higher risks of cardiovascular mortality. Thus, the finding that EPA may extend lag time to reduce platelet aggregation in males is indeed a novel and highly significant observation. Simultaneously, platelet-derived MP activity was reduced in parallel with platelet aggregation and the extension of lag time in males supplemented with EPA only. With respect to procoagulant activity, lag time is reflective of prothrombin time and this is important given that MP activity is initiated following the exposure of PS where prothrombin is cleaved into thrombin [450]. Indeed EPA has been reported to be incorporated in PS following EPA supplementation in healthy male subjects [456]. Furthermore, Throngren and co-workers have also reported that an EPA enriched diet significantly increased prothrombin time and bleeding time in a population of healthy male individuals [295]. The increased lag time in parallel with the decreased MP activity and platelet aggregation suggest a potential pathway whereby EPA may reduce platelet aggregation via incorporation of EPA into PS or via other means of affecting the assembly of the prothrombinase complex. Consistently reflecting these findings, longer term supplementation of 4 weeks with EPA-rich oils reduced prothrombin activity and factor V activity in male subjects only (Chapter 5).

Factor V is an enzymatically inactive protein that serves as a cofactor for activated FX(a) to enable the assembly of the prothrombinase complex on the surface of
activated platelets [84, 486]. This complex is initiated when activated platelet membranes expose anionic membrane phospholipid which permits the binding of Factor V with FXa on the platelet surface [499]. Subsequently, prothrombin that is bound to glycoprotein GPIIb/IIIa complex within the membrane is cleaved by FXa resulting in the release of thrombin [82]. Prothrombin has been reported to be correlated more closely with cardiovascular risk factors than fibrinogen and factor VII, and consistently reflected the difference in cardiovascular risk when correlated with risk factors which have markedly different effects between the sexes [500]. The production of thrombin from prothrombin in turn stimulates platelet activation [70] which is mediated by adhesive proteins such as vWF to allow platelet interaction. The outcome is cross-linking of GPIIb/IIIa receptors on adjacent platelets and subsequent platelet aggregation [501]. Essentially, the adhesion of platelets to collagen mediated by vWF activates platelets for aggregation and proteins for coagulation. Indeed increases in prothrombin, FV and vWF serve as potential risk factors for thrombotic events. Elevated prothrombin and FV activity levels are associated with arterial and venous thrombosis [251, 487-490] and reduction of plasma vWF activity decreases risk in cardiac patients [491]. Though the decrease in vWF antigen levels in our male cohort did not reach significance when compared between treatment groups, a significant gender x treatment effect was apparent ($P=0.034$) such that EPA supplementation reduced vWF levels in males while DHA reduced vWF levels in females.

Incorporation of EPA/DHA in platelet membrane alters the phospholipid composition inducing subtle changes both in the inner and the outer plasma membrane. This is of significance given that the outer platelet membrane provides the catalytic surface for the binding of FV, cleavage of prothrombin and subsequent adhesion and aggregation of platelets. The process of hemostasis and thrombus formation depends on the fine balance between the coagulation and fibrinolysis systems. High levels of PAI-1 lead to a reduction in plasmin degradation of fibrin promoting thrombosis [472]. Thus fibrinolysis, the final stage in hemostasis has been considered a target treatment approach to enhance dissolution of the thrombus. Consistently, chapter 5 demonstrated not only the reduction of platelet aggregation, procoagulant activity and coagulation factors in males supplemented with EPA, but also the gender-dependent reduction of PAI-1 levels in this cohort. While dietary
DHA had no effect on the hemostatic parameter in healthy males, in females supplementation markedly reduced platelet aggregation activity however the exact mechanism remains unclear. Though other parameters were not significantly altered in females consuming the DHA-rich oils, the coagulation profile was marginally influenced with a uniform decreasing trend. In contrast, the EPA-rich oils were unique in decreasing the coagulant potential in male subjects with a marked anti-platelet and modest anti-coagulant effects.

The novel findings from these series of research trajectories demonstrated that while DHA-supplementation inhibits platelet aggregation in females by mechanisms warranting further investigation; EPA-rich oil supplementation reduces thrombotic disease risk in males by:

- Inhibition of platelet aggregation
- Extension of lag time
- Reduction of platelet-derived microparticle activity
- Decreased vWF levels
- Reduction of prothrombin and factor V levels
- Decreased PAI-1 levels

The observed effects of EPA may be owing to the possibility that EPA is more effectively incorporated into platelets in males than females, while females are able to retain DHA more efficiently. Indeed females have a greater capacity to synthesize DHA compared to males [200, 346, 457]. Oestrogens have been shown to increase DHA synthesis, while testosterone has been reported to decrease DHA concentrations [337]. Higher synthesis and/or retention of DHA in females compared to males may explain the observed greater inhibition of platelet aggregation in females. There has been no reported association between DHA with prothrombin or bleeding time, rather the anti-aggregatory effects of DHA have been reported to be independent of prothrombin fragments [280, 282], which may explain why lag time was not related to the reduced aggregation observed with DHA in female subjects.

The lack of effect observed in males with DHA supplementation may be explained by sex differences in LCn-3PUFA metabolism whereby males are unable to retain sufficient DHA in comparison to females. The ability of LCn-3 PUFA to modify and
improve plasma lipid profiles offers vascular and cardio protection. However, the metabolic capacity of an individual to biosynthesize and mobilize EPA is important to enable the utilization of these fatty acids. As described in Chapter 1 (section 1.3.3), biosynthesis of LCn-3PUFAs are different in men and women [200] and more recently, distribution, interconversion, and dose response of n–3 fatty acids in humans have been suggested to be influenced by gender [206]. In line with this, Chapter 5 demonstrated a gender x treatment interaction whereby plasma DHA levels increased to a significantly greater extent in females than males (148% vs. 64% respectively) following DHA-rich oil supplementation. Furthermore, testosterone levels were inversely correlated with plasma DHA levels post DHA supplementation but positively associated to changes in platelet aggregation post DHA supplementation. Other investigators have also reported observations that testosterone administration reduce DHA levels [337] and increase platelet aggregation responses in healthy males [333]. In addition, the observed decrease in prothrombin and factor V activity was associated with lower oestradiol levels. The sex-dependent results on fatty acid uptake in studies herein parallel the gender-specific decrease in platelet and procoagulant activity and collectively reflect the reported literature. This suggests that higher testosterone levels may regulate the retention (reduced uptake and/or increased oxidation) of DHA from the supplement, resulting in only limited inhibition of platelet aggregation in the males.

6.2. Research strength and limitations

The inconsistency in the available fish and platelet aggregation literature has been compounded by methodological weaknesses related to study design, sample size, instrumentation, fish oil concentration ratios and lack of pharmacokinetic studies which preclude definite conclusions. The strength of the research in this thesis has been greatly enhanced by addressing and controlling for these entire variables. The use of the impedance aggregometer to measure platelet aggregation made possible the assessment of platelet function in whole blood, in the presence of all the formed elements of blood, without centrifugation or blood cell counts. Adopting this method in the conducted research allowed the study of platelets in their physiological milieu.
where the whole population of platelets were evaluated, rather than the artificial selection of larger platelet populations in PRP used in other studies [275, 280, 291]. The volume required and sample preparation were also greatly reduced hence preserving labile modulators such as prostacyclin and thromboxane A\textsubscript{2} resulting in a testing environment proven to be more sensitive to the effects of platelet abnormalities [412, 413]. With respect to the \textit{in vitro} investigation (Chapter 3), the contribution of blood cells to assess the anti-platelet activity of EPA vs. DHA permitted a better extrapolation of the results to \textit{in vivo} situations. In the assessment of the acute effects of EPA vs. DHA (Chapter 4), the avoidance of the processing of the blood sample allowed it to be more likely to represent the \textit{in vivo} status of platelets at the time the blood sample was obtained [502]. Furthermore, the use of collagen as an agonist to stimulate platelet aggregation in the studies is more reflective of primary hemostasis physiologically, because \textit{in vivo}, damaged vascular endothelial surface exposes subendothelial collagen fibres, initiating platelet aggregation [105].

With respect to dietary intervention, the fish oil supplements used in these studies were pure, natural oils opposed to the earlier studies using ethyl esters [202, 290, 361] which are less bioavailable [503] and are more susceptible to oxidation [504, 505]. Most importantly, these research studies have been the first controlled human trials directly comparing identical intakes of EPA with DHA-rich oils in a sex-stratified cohort. In Study 2, the effect of inter-individual variability was minimized by directly comparing the hemostatic responses from the same participants when supplemented with EPA vs. DHA vs. Placebo. Study 3 employed a double-blinded randomized placebo controlled trial with sex stratification that ensured there was no study bias. However, initially the study was powered to investigate the effects of EPA versus DHA only; therefore Study 2 may have been underpowered to detect sex-specific changes. The study population also involved women of post-menopausal status with lower levels of oestrogens which were approximately 30\% of the female cohort. Additional studies comparing the effects of fish oil supplementation in pre- and post-menopausal women are warranted. Finally, future studies examining EPA and/or DHA in males vs. females should be extended to populations or individuals with abnormal platelet function when examining the association between dietary LCn-3PUFA and biomarkers of thrombotic disease risk.
Future research efforts should be designed to provide more gender-specific information on treatment disposition and clinical effect. In addition, further studies with a larger sample size carried out over a longer period may be necessary to substantiate these findings.

6.3. Implications of the body of research

The regulation of platelet and procoagulant activity is critical for cardiovascular health as increased procoagulant tendency is a major risk factor for the development of thrombotic diseases such as stroke and myocardial infarction. These nutritional approaches may provide a useful preventive approach or an adjunct to current pharmacological treatments for these thrombotic diseases. The work embodied in this thesis has demonstrated interplay of distinct gender differences from baseline hemostatic markers to platelet characteristics and from LCn-3PUFA plasma fatty acid metabolism to membrane phospholipid incorporation. Given that sex hormones contribute substantially to the platelet response and ultimately cardiovascular outcome, these gender-specific results have implications for the use of EPA and DHA as a safe and effective prophylaxis for thrombotic disease. While more studies are required to clarify this aspect of LCn-3PUFA interactions with the hemostatic system in cardiovascular patients, this gender-dependent regulation may have important implications for understanding the basis of the gender gap in thrombotic complications. Given the toll taken by thrombotic diseases, a mechanistic understanding of how the role of gender functions as a disease modifier would be highly desirable. This thesis demonstrates that a single oral 1g dose of EPA or DHA reduces platelet aggregation up to 24 hours; the anti-thrombotic effects of which are maintained over 4 weeks with supplementation. The significance of these findings provides insight into the intricate mechanism whereby EPA and DHA exert their effects. In addition, applicably; these novel findings allows for optimal cardio-protection tailored for both gender groups offering a safe and non-pharmacological approach. The findings generated from this body of research may assist in establishing risk stratification by the ‘EPA+DHA’ level according to gender. With
respect to thrombotic disease risk, males would likely benefit more from supplementation with EPA while females are more responsive to DHA.

### 6.4. Final conclusion

The ongoing clinical research may potentiate the development of more potent anti-platelet drugs; however a non-pharmacological approach to lower platelet function may be another intriguing prospective. Along with the emerging entity of drug resistance, natural anti-thrombotic agents that influence platelet function are of interest for primary prevention of cardiovascular disease. As CVD continues to be an overwhelming burden, there is an urgent need to develop safe and efficacious, non-pharmacological approaches to prevent platelet hyperactivity to ultimately reduce the risk of CVD.

The overall findings from this body of research provide a first indication that gender contributes to differential platelet and procoagulant responses according to EPA or DHA supplementation. The associations between sex-hormonal levels with platelet aggregation and procoagulant activity following LCn-3PUFA supplementation provide substantial evidence that validate the research hypothesis that forms this thesis. Gender-specific responses exist in platelet aggregation following LCn-3PUFA supplementation. Inhibition of platelet aggregation and hemostatic markers by EPA is limited to males only while the anti-aggregatory effects of DHA are unique to females. These results may explain the generated controversies surrounding the anti-aggregatory and anti-thrombotic effects. This knowledge should foster the development of anti-thrombotic therapy and assist in the evolution of fish oil research. In conclusion, the work embodied in this thesis provides substantial evidence that the effects of EPA and DHA on platelet and procoagulant activity are apparent. These anti-thrombotic effects of EPA and DHA are neither shared nor complementary; rather they are gender-specific.
References


137. Trigui, N., et al., *Molecular characterization of a novel mutation in the factor XIII A subunit gene associated with a severe defect and an adulthood*


312. Kjeldsen, S.E., et al., Influence of gender and age on preventing cardiovascular disease by antihypertensive treatment and acetylsalicylic


Appendices
Appendix One: Study 1 Information Statement & Consent Form

Information Statement

Docosapentaenoic acid (DPA) is a functional long-chain omega-3 polyunsaturated fatty acid

Investigators: Professor Manohar Garg, Miss Melinda Phang

Thank you for your interest in our research on omega-3 fatty acids and platelet function. This study is being carried out by researchers from the School of Biomedical Sciences at the University of Newcastle.

Why is the research being done?

The purpose of this research is to examine the effects of different fish oils that can decrease the activation of platelets involved in blood clotting. There is evidence that supplementation with fish oil can improve platelet function (involved with blood clotting).

Who can participate?

To take part in this study you must be:

- Healthy male or female.
- Aged above 18 years at initial assessment.

You may NOT be eligible to participate if you are of the following:

- Diagnosed with Non-Insulin Dependent Diabetes Mellitus (NIDDM) - type 2
- Diagnosed with any cardiovascular, haematological or gastrointestinal disorder.
- Have a body mass index (BMI) greater than 35.
- Currently taking aspirin or other drugs which affect blood coagulation (your doctor will know this).
- Currently taking any nonsteroidal anti-inflammatory drugs.
- Taken fish oil (omega-3) as supplements within the previous 6 weeks.
- Have eaten high levels of tomato products within the previous 6 weeks.

Participation in this research is voluntary and entirely your choice. Only those people who have given their informed consent will be included in this project. If you do
decide to participate, you may withdraw your consent for participation in this research project at any time without giving a reason.

**What would you be asked to do?**

If you agree to participate, we will be asking you to do the following:

- Follow a diet that is low in tomato and fish products for 1 week.
- Complete a brief medical questionnaire.
- Take body weight and height measurements.
- Recall food and beverages you have consumed over the last 24 hours.
- Donate a fasting blood sample (20mL) on 1 occasion.

If you agree to participate, we will be asking you to attend the University of Newcastle on 1 occasion for approximately 30 minutes to obtain this information and collect a blood sample.

**What are the risks and benefits of participating?**

There are some risks of having blood collected, including bruising or bleeding from the collection site, as well as fainting and dizziness. However, these risks are minimal and a qualified and experienced venepuncturist will take your blood in order to minimise these risks. There is no direct benefit by participating in this study, however you may request information about your health from any examinations and laboratory tests that are undertaken in this study. Feedback of results from the investigation and any other information regarding to the outcome of this research will be made available to you when the study is completed.

**How will the information collected be used?**

Results of this research will be published in scientific journals and will be available for participants to access. **Individual participants will not be identified** in any reports arising from the project.

**How will your privacy be protected?**

Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor.

Information collected for this project will be stored in a database at the University of Newcastle only identifiable by a participant identification number with no other identifying details. The database will be stored in a password protected computer file on a computer that is kept in a locked room. All data for the study will be kept by the Chief Investigator for the period of 15 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.
What do you need to do to participate?

Please read this Information Statement and be sure you understand its contents before you consent to participating. If there is anything you do not understand, or you have questions please contact the study principle investigator.

If you would like to participate, please complete the attached Consent Form and return it to the investigators at the University of Newcastle.

Further information

Further information about this or any other research project undertaken in the department at the University of Newcastle can be obtain from either the Principle Investigator of the study, Professor Manohar Garg or the study co-investigator Miss Melinda Phang (4921 5636).

Thank you for considering this invitation to participate in research undertaken at the University of Newcastle.

Professor Manohar Garg
Principle Investigator,
University of Newcastle

Miss Melinda Phang
Study Co-investigator,
University of Newcastle

Complaints

This research has been reviewed and approved by the University of Newcastle Human Research Ethics Committee (Approval No. H-2008-0149). The University of Newcastle requires that all participants are informed that should you have any concerns about your rights as a participant in research undertaken at the University of Newcastle, or you have a complaint about the manner in which information is collected, it may be given to the study researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, telephone (02) 49216333, email Human-Ethics@newcastle.edu.au.

Professor Manohar Garg
PhD, MND, MSc (Biochem), BSc (Hons)
Director, Nutraceuticals Research Group
School of Biomedical Sciences
University Drive, Callaghan
NSW 2308 Australia
Phone: +61 2 4921 5647
Fax: +61 2 4921 2028
Email: manohar.garg@newcastle.edu.au
Participant Consent Form

Docosapentaenoic acid (DPA) is a functional long-chain omega-3 polyunsaturated fatty acid

I give my consent to participate in the above research project designed to test the effectiveness of omega-3 fatty acids (fish oil) on platelet function in blood clotting. I understand that the following information has been provided to me in the Information Statement, a copy of which I have retained;

- I understand that my participation is voluntary and entirely my choice.
- I understand I can withdraw my consent for participation at any time and do not have to give any reason for withdrawing.
- I understand that I will be required to follow a diet that is low in tomato and fish products for 1 week.
- I understand that I will be required to give blood (max 20mL) on 1 occasion after an overnight fast.
- I understand that I am required to complete a medical questionnaire, provide a recall on foods/drinks consumed over 24 hours and allow body weight and height measurements.
- I understand that my personal information will remain confidential to the researchers.

Signed by Participant

Print Name: ____________________________
Signed: ____________________________
Date: ___ / ___ / _____

Signed by Study Investigator

Name: ____________________________
Signed: ____________________________
Date: ___ / ___ / _____

Professor Manohar Garg
PhD, MND, MSc (Biochem), BSc (Hons)
Director, Nutraceuticals Research Group
School of Biomedical Sciences
University Drive, Callaghan
NSW 2308 Australia
Phone: +61 2 4921 5647
Fax: +61 2 4921 2028
Email: manohar.garg@newcastle.edu.au
Appendix Two: Study 2 Information Statement & Consent Form

FACULTY OF HEALTH

Information Statement

Docosapentaenoic acid (DPA) is a functional long-chain omega-3 polyunsaturated fatty acid

Investigators: Professor Manohar Garg, Miss Melinda Phang

Thank you for your interest in our research on omega-3 fatty acids and platelet function. This study is being carried out by researchers from the School of Biomedical Sciences at the University of Newcastle.

Why is the research being done?

The purpose of this research is to examine the effects of different fish oil (omega-3 fatty acids) concentrates that can decrease the activation of platelets involved in blood clotting. There is evidence that supplementation with fish oil can improve platelet function (involved with blood clotting).

Who can participate?

To take part in this study you must be:

- Healthy male or female.
- Aged above 18 years at initial assessment.

You may NOT be eligible to participate if you are of the following:

- Diagnosed with Non-Insulin Dependent Diabetes Mellitus (NIDDM) - type 2 diabetes.
- Diagnosed with any cardiovascular, haematological or gastrointestinal disorder.
- Have a body mass index (BMI) greater than 35.
- Currently taking aspirin or other drugs which affect blood coagulation (your doctor will know this).
- Currently taking any nonsteroidal anti-inflammatory drugs.
- Taken fish oil (omega-3 fatty acids) as supplements within the previous 6 wks.
- Have eaten high levels of tomato products within the previous 6 weeks.
- Have an allergy to fish products

Participation in this research is voluntary and entirely your choice. Only those people who have given their informed consent will be included in this project. If you do
decide to participate, you may withdraw your consent for participation in this research project at any time without giving a reason.

**What would you be asked to do?**

Participation in this study will involve receiving 3 cycles of nutritional supplements and blood tests. If you agree to participate, we will be asking you to do the following:

- Follow a diet that is low in tomato and fish products for 1 week.
- Complete a brief medical questionnaire.
- Take body weight and height measurements
- Recall food and beverages you have consumed over the last 24 hours
- Receive the following dietary supplements with a washout period of at least one week between receiving each of the supplements:
  - 1 x 2g dose (1/2 tsp) fish oil concentrate containing low DPA
  - 1 x 2g dose (1/2 tsp) fish oil concentrate containing high DPA
  - 1 x 2g dose (1/2 tsp) placebo (olive oil)
- Donate blood (20 mL) on 4 occasions: after overnight fast then 2, 5 and 24 hours after consumption of the supplement.
- Follow a diet that is low in tomato and fish products for 1-2 weeks between consumption of each dietary supplement.

If you agree to participate, for each separate trial of the supplements we will be asking you to attend the University of Newcastle on 3 occasions for approximately 5 ½ hours each and on another occasion for approximately 20 minutes to obtain information and collect blood samples.

**What are the risks and benefits of participating?**

There are some risks of having blood collected, including bruising or bleeding from the collection site, as well as fainting and dizziness. However, these risks are minimal and a qualified and experienced venepuncturist will take your blood in order to minimise these risks.

The supplements will be provided at no cost to you. You may request information about your health from any examinations and laboratory tests that are undertaken in this study. Although there is evidence that fish oils will improve platelet function by reducing blood clotting, there is no guarantee that you will receive any benefit from the treatment. Feedback of results from the investigation and other information regarding to the outcome of the research will be made available to you and will be posted as an individual letter at the completion of the study.

**How will the information collected be used?**

Results of this research will be published in scientific journals and will be available for participants to access. **Individual participants will not be identified** in any reports arising from the project.
How will your privacy be protected?

Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor. Information collected for this project will be stored in a database at the University of Newcastle only identifiable by a participant identification number with no other identifying details. The database will be stored in a password protected computer file on a computer that is kept in a locked room. All data for the study will be kept by the Chief Investigator for the period of 15 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.

What do you need to do to participate?

Please read this Information Statement and be sure you understand its contents before you consent to participating. If there is anything you do not understand, or you have questions please contact the study principal investigator.

If you would like to participate, please complete the attached Consent Form and return it to the investigators at the University of Newcastle.

Further information

Further information about this or any other research project undertaken in the department at the University of Newcastle can be obtain from either the Principle Investigator of the study, Professor Manohar Garg or the study co-investigator Miss Melinda Phang (4921 5636).

Thank you for considering this invitation to participate in research undertaken at the University of Newcastle.

Professor Manohar Garg
Principle Investigator,
University of Newcastle

Miss Melinda Phang
Study Co-investigator,
University of Newcastle

Complaints

This research has been reviewed and approved by the University of Newcastle Human Research Ethics Committee (Approval No. H-2008-0149). The University of Newcastle requires that all participants are informed that should you have any concerns about your rights as a participant in research undertaken at the University of Newcastle, or you have a complaint about the manner in which information is collected, it may be given to the study researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, telephone (02) 49216333, email Human-Ethics@newcastle.edu.au.

Professor Manohar Garg
PhD, MND, MSc (Biochem), BSc (Hons)
Director, Nutraceuticals Research Group
School of Biomedical Sciences
University Drive, Callaghan
NSW 2308 Australia
Phone: +61 2 4921 5647; Fax: +61 2 4921 2028
Email: manohar.garg@newcastle.edu.au
Participant Consent Form

Docosapentaenoic acid (DPA) is a functional long-chain omega-3 polyunsaturated fatty acid

I give my consent to participate in the above research project designed to test the effectiveness of omega-3 fatty acids (fish oil) on platelet function in blood clotting. I understand that the following information has been provided to me in the Information Statement, a copy of which I have retained:

- I understand that my participation is voluntary and entirely my choice.
- I understand I can withdraw my consent for participation at any time and do not have to give any reason for withdrawing.
- I understand that I will be required to follow a diet that is low in tomato and fish products for 1 week.
- Receive the following dietary supplements with a washout period of at least one week between receiving each of the supplements:
  - 1 x 2g dose (1/2 tsp) fish oil concentrate containing low DPA
  - 1 x 2g dose (1/2 tsp) fish oil concentrate containing high DPA
  - 1 x 2g dose (1/2 tsp) placebo (olive oil)
- I understand that for each trial supplement I will donate blood (20 mL) on 4 occasions: after overnight fast then 2, 5 and 24 hours after consumption of the supplement.
- I understand that I am required to have a washout period of at least one week between receiving each of the supplements.
- I understand that I am required to complete a medical questionnaire, provide a recall on foods/drinks consumed over 24 hours and allow body weight and height measurements.
- I understand that my personal information will remain confidential to the researchers.

Feedback of results from the investigation and other information regarding to the outcome of the research will be made available to me and will be posted as an individual letter at the completion of the study.

Signed by Participant

Print Name: ___________________________  Print Name: ___________________________
Signed: _______________________________  Signed: _______________________________
Date: ___ / ___ / _____  Date: ___ / ___ / _____

Professor Manohar Garg
PhD, MND, MSc (Biochem), BSc (Hons)
Director, Nutraceuticals Research Group
Phone: +61 2 4921 5647
Fax: +61 2 4921 2028, Email: manohar.garg@newcastle.edu.au
Appendix Three: Study 3 Information Statement & Consent Form

FACULTY OF HEALTH

Long chain omega-3 polyunsaturated fatty acids and platelet function

Investigators: Professor Manohar Garg, Ms Melinda Phang

Thank you for your interest in our research on omega-3 fatty acids and platelet function. This study is being carried out by researchers from the School of Biomedical Sciences & Pharmacy at the University of Newcastle, as part of the PhD studies of Melinda Phang under the supervision of Professor Manohar Garg.

Why is the research being done?

The purpose of this research is to examine how different fish oil (omega-3 fatty acids) concentrates can influence platelet activity and improve platelet function in males and females. There is evidence in the published literature that response to fish supplementation is influenced by a gender-specific manner. Moreover, fish oil supplements contain different concentration ratios of the active ingredients, EPA and DHA. This research project will determine how gender influences fish oil supplementation so that males and females can receive maximum benefit against cardiovascular disease risk.

Who can participate?

To take part in this study you must be:

- Healthy male or female.
- Aged above 18 – 75 years at initial assessment.

You may NOT be eligible to participate if you are of the following:

- Diagnosed with Non-Insulin Dependent Diabetes Mellitus (NIDDM) - type 2
- Diagnosed with any cardiovascular, haematological or gastrointestinal disorder.
- Have a body mass index (BMI) greater than 35 [BMI = weight (kg)/ height (m)$^2$]
- Currently taking aspirin or other drugs which affect blood coagulation (your doctor will know this).
- Currently taking any nonsteroidal anti-inflammatory drugs.
- Have taken fish oil (omega-3 fatty acids) as supplements within the previous 6 weeks.
- Have eaten high levels of tomato products within the previous 6 weeks.
- Have an allergy to fish products

Participation in this research is voluntary and entirely your choice. Only those people who have given their informed consent will be included in this project. If you do
decide to participate, you may withdraw your consent for participation in this research project at any time without giving a reason.

**What would you be asked to do?**

Participation in this study will involve receiving daily supplementation with nutritional supplements or a placebo (a capsule that contains no active ingredients) over 4 weeks and 2 blood tests. If you agree to participate, we will be asking you to do the following:

- Follow a diet that is low in tomato and fish products for 1 week.
- Complete a brief medical questionnaire.
- Take body weight and height measurements
- Recall food and beverages you have consumed over the last 24 hours
- Consume either one of the following dietary supplements daily over a 4 week period:
  - 1 x 2g dose fish oil capsules containing high EPA
  - 1 x 2g dose fish oil capsules containing high DHA
  - 1 x 2g dose placebo capsules (Sunola oil)
- Donate blood (25 mL) on 2 occasions: after an overnight fast before and after an overnight fast following 4 weeks of supplementation.
- Follow a diet that is low in tomato and fish products during the study period.

If you agree to participate, we will be asking you to attend the University of Newcastle on 2 occasions for approximately 30 minutes on each occasion to obtain information and collect blood samples.

**What are the risks and benefits of participating?**

There are some risks of having blood collected, including bruising or bleeding from the collection site, as well as fainting and dizziness. However, these risks are minimal and a qualified and experienced pathology collector will take your blood in order to minimise these risks.

The supplements will be provided at no cost to you. You may request information about your health from any examinations and laboratory tests that are undertaken in this study. Although there is evidence that fish oils will improve platelet function by reducing blood clotting, there is no guarantee that you will receive any benefit from the treatment. Feedback of results from the investigation and other information regarding to the outcome of the research will be made available to you and will be posted as an individual letter at the completion of the study.

**How will the information collected be used?**

Results of this research will be published in scientific journals and be reported in Melinda Phang’s PhD thesis. Individual participants will not be identified in any reports arising from the project.

**How will your privacy be protected?**
Your information will be treated with the same respect for privacy and confidentiality as is undertaken for all medical information collected about you during your visits to your local doctor.

Information collected for this project will be stored in a database at the University of Newcastle only identifiable by a participant identification number with no other identifying details. The database will be stored in a password protected computer file on a computer that is kept in a locked room. All data for the study will be kept by the Chief Investigator for a minimum period of 15 years following the completion of the study. Only staff of the University of Newcastle conducting this research will have access to this information.

**What do you need to do to participate?**

Please read this Information Statement and be sure you understand its contents before you consent to participating. If there is anything you do not understand, or you have questions please contact the study principle investigator.

If you would like to participate, please complete the attached Consent Form and return it to the investigators at the University of Newcastle.

**Further information**

Further information about this or any other research project undertaken in the department at the University of Newcastle can be obtain from either the Principle Investigator of the study, Professor Manohar Garg or the Study Co-investigator Miss Melinda Phang (4921 5636), Email: Melinda.phang@newcastle.edu.au

Thank you for considering this invitation to participate in research undertaken at the University of Newcastle.

__________________________
Professor Manohar Garg
Principle Investigator,
University of Newcastle

__________________________
Ms Melinda Phang
Study Co-investigator,
University of Newcastle

Complaints

This research has been reviewed and approved by the University of Newcastle Human Research Ethics Committee (Reference No. H-2010-0022). The University of Newcastle requires that all participants are informed that should you have any concerns about your rights as a participant in research undertaken at the University of Newcastle, or you have a complaint about the manner in which information is collected, it may be given to the study researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, telephone (02) 49216333, email Human-Ethics@newcastle.edu.au.
Long chain omega-3 polyunsaturated fatty acids and platelet function

I give my consent to participate in the above research project designed to test the efficacy of omega-3 fatty acids (fish oil) on platelet function in blood clotting. I understand that the following information has been provided to me in the Information Statement, a copy of which I have retained:

• I understand that my participation is voluntary and entirely my choice.
• I understand I can withdraw my consent for participation at any time and do not have to give any reason for withdrawing.
• I understand that I will be required to follow a diet that is low in tomato and fish products for 1 week.
• Consume either one of the following dietary supplements daily over a 4 week period:
  1 x 2g dose fish oil capsules containing high EPA
  1 x 2g dose fish oil capsules containing high DHA
  1 x 2g dose placebo capsules (Sunola oil)
• I understand that I will donate blood (25 mL) on 2 occasions: before and after an overnight fast following 4 weeks of supplementation.
• I understand that I am required to complete a medical questionnaire, provide a recall on foods/drinks consumed over 24 hours and allow body weight and height measurements.
• I understand that my personal information will remain confidential to the researchers.

Feedback of results from the investigation and other information regarding to the outcome of the research will be made available to me and will be posted as an individual letter at the completion of the study.

Signed by Participant

Print Name: ____________________________
Signed: ____________________________
Date: ___ / ___ / _____

Signed by Study Investigator

Print Name: ____________________________
Signed: ____________________________
Date: ___ / ___ / _____

Professor Manohar Garg
PhD, MND, MSc (Biochem), BSc (Hons)
Director, Nutraceuticals Research Group
Phone: +61 2 4921 5647, Fax: +61 2 4921 2028
Email: manohar.garg@newcastle.edu.au
Appendix Four: Study 1 & 2 Participant Assessment Criteria

Participant Assessment Criteria

Participant ID Number: ____________ Trial Day: __________ Date: __/__/__

Inclusion Criteria

- Healthy male or female
- Are aged 18 years or above (DOB: __/__/__)

Exclusion Criteria

- Diagnosed with Non-Insulin Dependent Diabetes (NIDDM) - type 2
- Diagnosed with insulin resistance or impaired glucose tolerance.
- Diagnosed with any cardiovascular, haematological or gastrointestinal disorder.
- Have a body mass index (BMI) greater than 35
- Currently taking aspirin or other drugs which affect blood coagulation
- Currently taking any nonsteroidal anti-inflammatory drugs
- Have taken fish oil (omega-3 fatty acids) as supplements within the previous 6 weeks.
- Eaten high level of tomato products within the previous 6 weeks
- Allergy to fish products

Criteria assessed by: __________________ Date: __/__/__
Reviewed by Investigator: __________________ Date: __/__/__
### Participant Assessment Criteria

**Participant ID Number:** ____________ **Trial Day:** ______ **Date:** ___/___/____

#### Inclusion Criteria (must be either one)

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
</tbody>
</table>

- Healthy female aged 18 – 45 yrs (DOB:__/__/___)
- Healthy female aged 55 – 75 yrs (DOB:__/__/___)
- Are you of pre-menopausal status
- Are you of post-menopausal status

#### Exclusion Criteria

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
<tr>
<td><img src="false" alt=" " /></td>
<td><img src="false" alt=" " /></td>
</tr>
</tbody>
</table>

- Diagnosed with Non-Insulin Dependent Diabetes (NIDDM) - type 2
- Diagnosed with insulin resistance or impaired glucose tolerance.
- Diagnosed with any cardiovascular, haematological or gastrointestinal disorder
- Have a body mass index (BMI) greater than 35
- Taking aspirin or other drugs which affect blood coagulation
- Currently taking any nonsteroidal anti-inflammatory drugs
- Taking oral contraceptives or on hormone replacement therapy (HRT)
- Have taken fish oil (omega-3 fatty acids) as supplements within the previous 6 weeks.
- Have eaten high levels of tomato products within the previous 6 wks
- Allergy to fish products

---

**Criteria assessed by:** _________________________________ **Date:** __/__/__

**Reviewed by Investigator:** _______________________________ **Date:** __/__/__
Appendix Six: Pre-trial Medical Questionnaire

Professor ML Garg
Nutraceuticals Research Group
School of Biomedical Sciences
Telephone (02) 4921 5647

Pre-trial Medical Questionnaire

Participant ID Number: _______________ Date: ___/___/___

Date of birth: ___/___/___  Sex: ______ Height: ______cm  Weight: ______kg

1. Please list all current medical conditions (conditions which you are presently receiving treatment or advice):

Condition: _______________  Condition: _______________
Year diagnosed: _______________  Year diagnosed: _______________
Condition: _______________  Condition: _______________
Year diagnosed: _______________  Year diagnosed: _______________

2. Have you ever had (please circle):

A stroke? Yes / No  Angioplasty? Yes / No
A heart attack? Yes / No  By-pass surgery? Yes / No
Angina? Yes / No  Other heart/vascular surgery Yes / No
Other heart-related problems? Yes / No

3. Please list all medications that you take as prescribed by a doctor:

Brand name: _______________  Brand name: _______________  Brand name: _______________
Dose: _______________  Dose: _______________  Dose: _______________
Frequency: _______________  Frequency: _______________  Frequency: _______________
4. Please list all over-the-counter medications you take regularly

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. Please list all vitamin, mineral, and/or herbal supplements you take regularly

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brand name</th>
<th>Dose</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. Please indicate (circle) the number of alcoholic beverages you normally consume per week.

0     1-3     4-7     8-10     >10

7. Do you smoke? Yes / No

8. Please indicate (circle) how much exercise you normally conduct each week.

<table>
<thead>
<tr>
<th>Light (1-2 times/week)</th>
<th>Moderate (2-3 times/week)</th>
<th>Heavy (4-5 times week)</th>
<th>Very Heavy (every day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. Please indicate (circle) how much you exercised in the last 24 hours.

0     1-15 mins     16-30 mins     > 30 mins

Thank you for taking time to fill in this questionnaire
## Appendix Seven: 24 Hour Food Recall Form

---

**24 hour Food Recall**

Participant ID Number: __________ Trial Day: ______________ Date: ___/___/___

*Please list all foods and drinks consumed over the last 24 hours*

<table>
<thead>
<tr>
<th>Time</th>
<th>Food / Beverages</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |

|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |

|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |

|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |
|      |                  |          |
Instructions for recording food and drink consumption

You are being asked to record all foods and fluids you consumed in the last 24 hours. The information you record is very important to the success of this study. These instructions will help you to fill in your food and drink consumption in the most accurate manner.

Instructions:
- List the food and give a description, including method of cooking (e.g. grilled rump steak or fried rump steak). An example of how to record the information is given below.
- State the weight of the food or use standard household measure, such as a cup, a tablespoon, a teaspoon.
- Specify brand names of products for processed foods (e.g. Nestle diet yoghurt)
- Include supplements, and any condiments such as sauce or salad dressing

Example:

<table>
<thead>
<tr>
<th>Time/Meal (Breakfast/Lunch/Dinner)</th>
<th>Food/Drink + description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 am (Breakfast)</td>
<td>Kellogg’s corn flakes</td>
<td>1 cup</td>
</tr>
<tr>
<td></td>
<td>Dairy Farmer’s lite milk</td>
<td>100 mL</td>
</tr>
<tr>
<td></td>
<td>Boiled egg</td>
<td>2 medium</td>
</tr>
<tr>
<td></td>
<td>Black coffee – Nescafe instant</td>
<td>1 cup</td>
</tr>
<tr>
<td>10:30 am (Morning tea)</td>
<td>Tea/coffee</td>
<td>1 cup</td>
</tr>
<tr>
<td>12 noon (Lunch)</td>
<td>Salad sandwich – wholegrain bread</td>
<td>2 slices</td>
</tr>
<tr>
<td></td>
<td>Baby spinach leaves</td>
<td>1 cup</td>
</tr>
<tr>
<td></td>
<td>Tomato slices</td>
<td>3 slices</td>
</tr>
<tr>
<td></td>
<td>Edgell’s tinned beetroot</td>
<td>30 grams</td>
</tr>
<tr>
<td></td>
<td>Grated carrot</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red delicious apple</td>
<td>1 large</td>
</tr>
<tr>
<td></td>
<td>Nestle tropical fruit diet yoghurt</td>
<td>125 mL</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>500 mL</td>
</tr>
<tr>
<td>3:00 pm (Afternoon tea)</td>
<td>Diet coke</td>
<td>375 mL</td>
</tr>
<tr>
<td>6:00 pm (Dinner)</td>
<td>Pasta</td>
<td>2 cups</td>
</tr>
<tr>
<td></td>
<td>Salad – with tomato, lettuce ..... etc</td>
<td>List</td>
</tr>
<tr>
<td></td>
<td>Etc.</td>
<td></td>
</tr>
</tbody>
</table>
Appendix Eight: Statement of Contribution for Chapter One

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:


Melinda Phang contributed to the selection of studies in the review, drafting and final development of the manuscript. Sheryl Lazarus and Lisa Wood contributed to the review and manuscript development. Manohar L Garg contributed to the manuscript development within the capacity of the role of a PhD supervisor.

_________________________
Professor Manohar L Garg Date: 12/11/2012

_________________________
Dr Sheryl Lazaras Date: October 14, 2012

_________________________
Dr Lisa Wood Date: 15th October 2012

_________________________
Ms Melinda Phang Date: 9th November

_________________________
Professor John Rostas
Faculty of Health Assistant Dean (Research Training)

Date:
Appendix Nine: Statement of Contribution for Chapter One

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:

**Phang, M., Fry, M. & Garg, M.L; 'Omega-3 polyunsaturated fatty acids: Basic and Contemporary Research Issues'; Innovation in Healthy and Functional Foods (Ghosh/Das/Bagchi/Smarta; editors), CRC Press, September 2012; 419-434.**

Melinda Phang contributed to the selection of studies in the review, drafting and final development of the manuscript. Melissa Fry contributed to the review and manuscript development. Manohar L Garg contributed to the manuscript development within the capacity of the role of a PhD supervisor.

_________________________

Professor Manohar L Garg Date: 12/11/2012

_________________________

Miss Melissa Fry Date: 16th October 2012

_________________________

Ms Melinda Phang Date: 9th November 2012

_________________________

Professor John Rostas
Faculty of Health Assistant Dean (Research Training)

Date:
Appendix Ten: Statement of Contribution for Chapter Three

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:


Melinda Phang participated in the conception and design of the study, participant recruitment, sample and data collection and performed the statistical analysis. Manohar L Garg was involved in the design and coordination of the study, provided significant advice and consultation. Andrew J Sinclair was involved in the study design and provided expert advice during the course of the study.

_________________________
Professor Manohar L Garg
Date: 12/11/2012

_________________________
Professor Andrew J Sinclair
Date: 17/10/2012

_________________________
Ms Melinda Phang
Date: 9th November 2012

_________________________
Professor John Rostas
Faculty of Health Assistant Dean (Research Training)
Date:
Appendix Eleven: Statement of Contribution for Chapter Four

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:


Melinda Phang participated in the conception, study design, participant recruitment, sample and data analysis. Manohar L Garg and Lisa F Lincz were involved in the design and coordination of the study, provided significant advice and consultation. Andrew J Sinclair was involved in the study design and provided expert advice during the course of the study. All of the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript.

________________________________________________________________________

Professor Manohar L Garg                         Date: 12/11/2012

Professor Andrew J Sinclair                         Date: 17/10/2012

Dr Lisa F Lincz                                           Date:  

Ms Melinda Phang                                      Date: 9th November 2012

________________________________________________________________________

Professor John Rostas
Faculty of Health Assistant Dean (Research Training)

Date:
Appendix Twelve: Statement of Contribution for Chapter Four

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:


Melinda Phang participated in the conception, study design, participant recruitment, sample and data analysis. Lisa F Lincz and Michael Seldon were involved in the sample and data analysis and provided significant advice. Manohar L Garg was involved in the design and coordination of the study and provided significant advice and consultation. All of the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript.

_______________________
Professor Manohar L Garg Date: 12/11/2012

_________________________
Dr Lisa F Lincz Date:

_________________________
Dr Michael Seldon Date:

_________________________
Ms Melinda Phang Date: 9th November 2012

_________________________
Professor John Rostas
Faculty of Health Assistant Dean (Research Training)

Date:
Appendix Thirteen: Statement of Contribution for Chapter Five

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:


Melinda Phang contributed to the conception, study design, participant recruitment sample and data analysis. Manohar L Garg and Lisa F Lincz were involved the design and coordination of the study, provided significant advice and consultation. All of the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript.

________________________________________
Professor Manohar L Garg                        Date: 12/11/2012

________________________________________
Dr Lisa F Lincz                                 Date:

________________________________________
Ms Melinda Phang                                Date: 9th November 2012

________________________________________
Professor John Rostas
Faculty of Health Assistant Dean (Research Training)

Date:
Appendix Fourteen: Statement of Contribution for Chapter Five

I attest that Research Higher Degree candidate Melinda Phang contributed to the following publication:

**Phang, M., Scorgie, F.E, Seldon, M., Garg, M.L & Lincz, L; ‘Reduction of prothrombin and Factor V levels following supplementation with omega-3 fatty acids is gender-dependant: a randomised controlled study’ (Under review). Submitted for publication in ‘Thrombosis Research’ on November 19 2012.**

Melinda Phang participated in the conception, study design, participant recruitment, sample and data analysis. Lisa F Lincz and Fiona E Scorgie were involved in the sample and data analysis, Michael Seldon and Manohar L Garg was involved in the design and coordination of the study and provided significant advice and consultation. All of the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript.

_________________________
Professor Manohar L Garg  Date: 12/11/2012

_________________________
Dr Lisa F Lincz  Date:

_________________________
Dr Michael Seldon  Date:

_________________________
Ms Fiona E Scorgie  Date:

_________________________
Ms Melinda Phang  Date: 12th November 2012

_________________________
Professor John Rostas  
Faculty of Health Assistant Dean (Research Training)  
Date: