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

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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’ 14\textsuperscript{th} World Congress on Clinical Nutrition and 5\textsuperscript{th} International Congress on Cardiovascular Diseases (4-7 June 2009) Kosice, Slovakia.

2. **M. Phang**, M.L. Garg; ‘Redefining platelet aggregatory response to LC n-3PUFA, World Congress on Oils and Fats & 28\textsuperscript{th} ISF Congress’ (27 -30 Sep 2009) Sydney, Australia.


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Abbreviations

AA
Arachidonic acid

ALA
Alpha-linolenic acid

ACS
Acute coronary syndrome

ADP
Adenosine diphosphate

ALA
Alpha-linoleic acid

APTT
Activated partial thromboplastin time

AUC
Area under the curve

BMI
Body mass index

CAD
Coronary artery disease

CAM
Cellular adhesion molecule

CHD
Coronary heart disease

COX
Cyclo-oxygenase

CRP
C- reactive protein

CVD
Cardiovascular disease

DAG
Diaglycerol

DHA
Docosahexaenoic acid

DPA
Docosapentaenoic acid

DVT
Deep vein thrombosis

EPA
Eicosapentaenic acid

ETP
Endogenous thrombin potential

F
Factor

FA
Fatty acid

Fb
Fibrinogen

FO
Fish oil

GP
Glycoprotein

GPCR
G-protein coupled transmembrane receptors

IHD
Ischaemic heart disease

LA
Linoleic acid

LCFA
Long chain fatty acids

LCn-3PUFA
Long Chain omega-3 polyunsaturated fatty acids

LOX
Lipoxygenase

LRR
Leucine-rich repeated receptor
LT Leukotriene
MI Myocardial infarction
MP Microparticle
MUFA Monounsaturated fatty acid
n-3 Omega-3
n-6 Omega-6
PAF Platelet activating factor
PAI-1 Plasminogen activator inhibitor-1
PAR Protease activated receptor
PC Phosphatidyl choline
PCK Protein kinase C
PE Phosphatidyl ethanolamine
PE Pulmonary embolism
PG Prostaglandins
PGI Prostacyclin
PI Phosphatidyl inositol
PL Phospholipids
PLG Plasminogen
PS Phosphatidyl serine
P-sel P-selectin
PT Prothrombin time
RBC Red blood cell
SFA Saturated fatty acid
TF Tissue factor
TG Triglyceride
TNF-α Tumor necrosis factor-α
tPA Tissue plasminogen activator
TX Thromboxane
uPA Urokinase plasminogen activator
VTE Venous thromboembolism
vWF Von Willebrand factor
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:
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

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: