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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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.

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Melinda Phang

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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:

1. **Phang, M**., Garg, M.L. & Sinclair, A.J; 'Inhibition of platelet aggregation by long chain omega-3 polyunsaturated fatty acids is gender specific-Redefining platelet response to fish oils'. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2009; 81:35-40.

2. **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.

3. **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.

4. **Phang, M**., Sinclair, A.J., Lincz, L.F & Garg, M.L; ;Gender-specific inhibition of platelet aggregation following omega-3 fatty acid supplementation'; *Nutrition, Metabolism & Cardiovascular Diseases* 2012; 22:109-14.

5. **Phang, M.**, Lincz, L., Seldon, M & Garg, M.L; 'Acute supplementation with eicosapentaenoic acid reduces platelet microparticle activity in healthy subjects'; *Journal of Nutritional Biochemistry 2012*; 23:1128-33.

6. **Phang, M**., Lincz, L & Garg, M.L; 'Differential effects of eicosapentaenoic and docosahexaenoic acid on platelet aggregation and hemostatic in male versus female subjects' (Under review). Submitted for publication in 'Journal of Nutrition' on September 26 2012.

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

1. **M. Phang**, L.F Lincz, M.L. Garg; 'Differential effects of single oral dose of EPA or DHA rich oil supplementation on platelet aggregation in healthy human subjects'; Proceedings of the Nutrition Society of Australia 2009;33:37;Nutrition Society of Australia & NSNZ Joint Annual Scientific Meeting' (8-11 Dec 2009) Newcastle.

2. **M. Phang**, L.F Lincz, M Seldon, M.L. Garg; 'Platelet-derived microparticle activity is inhibited by omega-3 fatty acid supplementation'; Proceedings of the Nutrition Society of Australia 2010;34:5;Nutrition Society of Australia Annual Scientific Meeting (29 -3 Dec 2010) Perth, Australia.

3. **M. Phang**, L.F Lincz, M.L. Garg; 'Gender specific effects of long term dietary EPA and DHA supplementation in platelet aggregation in healthy human subjects'; Proceedings of the Nutrition Society of Australia 2011;35:92; Joint Nutrition Society of Australia & New Zealand ASM (29 – 3 Dec 2011), Queenstown, New Zealand.

4. **M. Phang**, L.F Lincz, M.L. Garg; 'Differential effects of eicosapentaenoic and docosahexaenoic acids on platelet aggregation and hemostatic markers in male versus female subjects', Joint Nutrition Society of Australia Annual Scientific Meeting (27 – 30 Nov 2012), Wollongong, Australia.

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.

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

3. **M. Phang**, L.F Lincz, M.L. Garg; 'Differential effects of dietary EPA or DHA supplementation on platelet aggregation in healthy male and female subjects' Australian Atherosclerosis Society 35th Annual Scientific Meeting (13 -16 Oct 2009) Melbourne, Australia.

4. **M. Phang**, L.F Lincz, M.L. Garg; 'Latest research on the effects of LC-PUFAs in human health and disease' Nestle Nutrition Update Day (22 May 2010) Royal North Shore Hospital, Sydney, Australia.

6. **M. Phang**, L.F Lincz, M Seldon, M.L. Garg; 'Gender specific role of n-3PUFA to inhibit microparticle activity in healthy humans' 15th World Congress on Clinical Nutrition (19-22 Sep 2010) El Sokhna, Egypt.

7. L.F Lincz, F. Scorgie, **M. Phang**, M.L. Garg , A. Enjeti, M. Seldon 'Variability in Platelet Microparticle Profile and Aggregation in Healthy Males and Females'; Annual Scientific Meeting of the Haematology Society of Australia and New Zealand, (17 – 20 Oct 2010) Auckland, New Zealand.

8. **M. Phang**, L.F Lincz, M Seldon, M.L. Garg 'Omega-3 polyunsaturated fatty acids inhibit platelet-derived microparticle activity in healthy human subjects' Joint Asia Pacific Society of Atherosclerosis & Vascular Biology and Australian

Atherosclerosis Society Annual Scientific Meeting (26 -29 Oct 2010) Cairns, Australia.

9. **M. Phang**, L.F Lincz, M.L. Garg; 'Dietary fish oil supplementation reduces platelet activity" Asian Congress of Nutrition 11th Scientific Meeting (13-16 July 2011), Singapore.
10. **M. Phang**, L.F Lincz, M.L. Garg; 'Inhibition of platelet aggregation by chronic dietary EPA or DHA is gender specific', Australian Atherosclerosis Society ASM (19-21 Oct 2011) Adelaide, Australia.
11. **M. Phang**, L.F Lincz, M.L. Garg; 'Eicosapentaenoic acid versus docosahexaenoic acid rich fish oils; effect on platelet physiological response', 'Food Structures, Digestion & Health Conference' (7-9 Mar 2012) Palmerston North, New Zealand.
12. **M. Phang**, L.F Lincz, M.L. Garg; 'Omega-3 polyunsaturated fatty acids and platelet function: sex dependence' Indian Society of Hypertension 22nd Annual Scientific Meeting (2-3 Nov 2012) Mumbai, India.
13. **M. Phang**, L.F Lincz, M.L. Garg; 'Efficacy of EPA and DHA-rich oils on platelet function in healthy men and women' Functional Foods, Nutraceuticals, Natural Health Products and Dietary Supplements (1-6 Dec 2012) Hawaii, USA.

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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:

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, 2012; 419-434.

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:***
>
> **Phang, M**., Garg, M.L & Sinclair, A.J. 'Inhibition of platelet aggregation by omega-3 polyunsaturated fatty acids is gender specific - Redefining platelet response to fish oils'; *Prostaglandins, Leukotrienes and Essential Fatty Acids* 2009:81:35-40.

(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:

Phang, M., Sinclair, A.J., Lincz, L.F & Garg, M.L; 'Gender-specific inhibition of platelet aggregation following omega-3 fatty acid supplementation'; *Nutrition, Metabolism & Cardiovascular Diseases* 2012:22:109-14.

Publication 5:

Phang, M., Lincz, L.F., Seldon, M & Garg, M.L; 'Acute supplementation with eicosapentaenoic acid reduces platelet microparticle activity in healthy subjects'; *Journal of Nutritional Biochemistry* 2012:23:1128-33.

(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:

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). Submitted for publication in Journal of Nutrition on October 26 2012.

Manuscript 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.