

**The Effects of Omega-3 Fatty Acids in an Ovine  
Model of Anthracycline-induced Non-ischaemic  
Cardiomyopathy**

**Angelo Carbone BSc**

Discipline of Medicine

Faculty of Health Sciences

The University of Adelaide, South Australia

&

Cardiovascular Research Centre

The Royal Adelaide Hospital, South Australia

A thesis submitted to the University of Adelaide  
in candidature for the degree of Master of Medical Science

July 2011

## **Abstract**

Anthracycline drugs, such as Doxorubicin (Adriamycin) (DOX), have been widely used since the 1960s for treatment of various forms of cancer. Despite their excellent anti-tumour effects, their clinical use may be complicated by various forms of cardiotoxicity, most notably dose dependent, non-ischaemic dilated cardiomyopathy (NICM) leading to congestive heart failure (CHF). Increasingly, different strategies have been devised in recent years to mitigate the adverse cardiovascular effects of anthracycline administration. However these have had variable success and the burden of anthracycline induced NICM remains substantial.

Marine derived omega-3 polyunsaturated fatty acids (PUFA) have been shown to have cardio-protective properties in a number of clinical settings. These include anti-arrhythmic, anti-inflammatory and anti-thrombotic properties and which are predominantly mediated by the longer chain omega-3 PUFA, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA).

Previously, a limited number of basic and small animal studies have evaluated the protective actions of omega-3 PUFA against anthracycline-induced cardiotoxicity, with mixed findings. Therefore the current study set out to expand on these results by investigating omega-3 PUFA supplementation in the translational setting of a large animal model of DOX-induced NICM.

Initially, a pilot study was performed to assess fatty acid bio-distribution in Merino wether sheep receiving marine fish oil (containing 300mg/mL EPA+DHA), administered by oral drenching of 23mL volumes three times

weekly for up to 20 weeks. Plasma and erythrocyte fatty acids were monitored serially and myocardial membrane concentrations were determined at study end. Systemic and myocardial uptake of long-chain omega-3 PUFA was demonstrated, with plasma, erythrocyte and myocardial concentrations increasing by two to three-fold from baseline levels ( $p < 0.05$ ).

For the main study, 17 age and weight-matched Merino wethers received fortnightly dosing with intracoronary DOX (1.2mg/kg for three doses) to induce cardiotoxicity. Animals were randomised to oral supplementation with fish oil (n=8) or olive oil placebo (n=9) commencing two to three weeks before DOX dosing and continued until 12 weeks after final DOX dose. Comparisons between the fish oil and placebo groups were made for left ventricular remodelling and function by cardiac magnetic resonance imaging (CMR), transthoracic echocardiography and histomorphometric analysis of myocardial fibrosis burden. Surprisingly, by comparison to placebo animals, sheep in the fish oil group showed greater decline in left ventricular ejection fraction (LVEF) ( $p < 0.05$ ), and greater end-diastolic and end-systolic dilatation after DOX ( $p < 0.05$ ). However, both groups demonstrated similar levels of left ventricular fibrosis, suggesting that the accentuation of systolic dysfunction observed in the omega-3 PUFA cohort was not mediated by excess myocardial collagen deposition.

In summary, this is the first large animal study to evaluate omega-3 PUFA supplementation in the setting of anthracycline cardiotoxicity. Despite augmenting circulating and tissue long-chain fatty acid levels, oral intake of fish-oil exacerbated cardiac remodelling induced by intracoronary DOX.

Given these new observational findings, we recommend deferring clinical investigation until further basic mechanistic studies can better define the interactions between fatty acids and cardiac biology in the presence of anthracycline exposure.

## **Declaration**

I declare that this thesis contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution to Angelo Carbone. To the best of my knowledge and belief, this thesis contains no material published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University of Adelaide Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Thesis Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Angelo Carbone, BSc.

July 2011

## **Acknowledgements**

I am sincerely grateful to the following staff members and departments for their support and assistance, without which this study would not have been possible.

First and foremost, my principal supervisor, Professor Stephen Worthley (Department of Medicine, University of Adelaide), who supported my enrolment into a postgraduate research program and provided extensive resources and academic support for this study. Professor Worthley has provided extensive and greatly appreciated support and encouragement towards my career and skills development in the field of cardiovascular research.

My co-supervisor, Dr Glenn Young, for his collaboration and previous work in evaluating the clinical effects of dietary omega-3 PUFA supplementation in the context of cardiovascular disease, that helped formulate the current study.

My great friend Dr Peter Psaltis (Cardiovascular Research Centre (CRC), Royal Adelaide Hospital (RAH)), who provided such great mentorship, support and superb interventional skills throughout the study. Peter performed each of the study's coronary angiograms and echocardiograms, which continued from his earlier work to develop and validate the DOX-ovine model. He provided extensive clinical peri-operative consultancy and study management advice, and significantly, whilst addressing his own challenges as a final year PhD student and planning a family move abroad.

To the staff of the Veterinary Services Division, Institute of Medical and Veterinary Science (IMVS) for their accommodating professional and provision of all anaesthetic procedures and veterinary management services relating to the study. In particular, the assistance and support of Melissa Gourlay, Adrian Hines, Jodie Dier, and Dr Tim Kuchel.

David Apps and Ella Zielinski of the Food and Nutrition Group, School of Agriculture, Food and Wine, University of Adelaide for conducting the fatty acid level analyses.

Dr Robert Metcalf (Rheumatology Unit and Cardiovascular Research Centre, Royal Adelaide Hospital) for his excellent guidance in relation to current clinical perspectives of omega-3 PUFA, his input into the study design, and for his coordination of the fatty acid samples analysis.

To Professor Michael James (Rheumatology Unit, Royal Adelaide Hospital and Department of Medicine, University of Adelaide) for his study management advice and insights into omega-3 PUFA uptake and metabolism.

To Ms Kerry Williams (Cardiovascular Investigations Unit (CVIU), RAH) for providing expert cardiac MR scanning services at all times of day, night and weekends.

To Professor Tony Thomas, (Pathology Department, Flinders Medical Centre, Bedford Park SA), for his generous and ready provision of histopathology consulting and processing services for this study.

To my great friend and colleague, Adam Nelson for volunteering so much of his own time to assist with after hours procedures and providing helpful advice on data management, analysis and presentation.

To Michael Weightman for performing LVEF and % Area Fibrosis analyses which provided pivotal outcome data for this study.

To Dr Michael Worthington (Cardiothoracic Surgery Department, Royal Adelaide Hospital), for his expert tuition and practical assistance with the pericardial window procedures.

To Dr Thomas Sullivan (Lecturer, Statistician, Discipline of Public Health, University of Adelaide) for providing statistical consultancy and analysis.

To Mr Anthony Brooks (Research Fellow, CRC RAH) for providing additional statistical analysis support.

To Ms Angie Hooper (Personal Assistant to Professor Worthley), for her helpful and professional administrative assistance and ongoing support throughout the study.



---

## Table of Contents

|                               |          |
|-------------------------------|----------|
| <b>Abstract</b> .....         | <b>2</b> |
| <b>Declaration</b> .....      | <b>5</b> |
| <b>Acknowledgements</b> ..... | <b>6</b> |

## Introduction

### 1.1 Cancer and Chemotherapy

|   |           |
|---|-----------|
| <b>1.1.1 Cancer</b> .....   | <b>18</b> |
| <i>1.1.1.1 Current Treatment Options</i> .....  | 18        |
| <b>1.1.2 Anthracyclines</b> .....   | <b>19</b> |
| <b>1.1.3 Cytotoxic Effects of Doxorubicin</b> .....                                     | <b>19</b> |
| <i>1.1.3.1 Generation of reactive oxygen species (ROS)</i> .....                        | 20        |
| <i>1.1.3.2 Antioxidant adjuvants to DOX therapy</i> .....                               | 22        |
| <i>1.1.3.3 Mechanisms of anthracycline-induced</i><br><i>anti-tumour activity</i> ..... | 22        |
| <i>1.1.3.4 Apoptosis</i> .....  | 23        |

### 1.2 Doxorubicin-induced Cardiomyopathy

|  |           |
|--|-----------|
| <b>1.2.1 Mechanisms of Anthracycline-induced</b><br><b>Cardiomyopathy</b> .....    | <b>25</b> |
| <i>1.2.1.1 Apoptosis and oxidative stress</i> .....                                | 25        |
| <i>1.2.1.2 Down-regulation of cardiac specific</i><br><i>muscle proteins</i> ..... | 26        |
| <i>1.2.1.3 Release of vasoactive substances</i> .....                              | 26        |
| <b>1.2.2 Cardiac Monitoring of Patients Receiving Anthracyclines</b> ...           | <b>28</b> |

|            |  |    |
|------------|--|----|
| <b>1.3</b> | <b>Treatment Strategies to Reduce Anthracycline-induced<br/>NICM</b>                                   |    |
|            | <i>1.3.1.1 Dosing regime</i> .....   | 29 |
|            | <i>1.3.1.2 Anthracycline analogues</i> .....   | 29 |
|            | <i>1.3.1.3 Liposomal preparations</i> .....  | 30 |
|            | <b>1.3.2 Cardioprotective Adjuncts</b>   |    |
|            | <i>1.3.2.1 Dexrazoxane</i> .....   | 30 |
|            | <i>1.3.2.2 Hematopoietic cytokines</i> .....   | 30 |
|            | <i>1.3.2.3 Antioxidants</i> .....  | 31 |
|            | <i>1.3.2.4 Improved prognosis with current generation<br/>heart failure medications</i> .....          | 31 |
| <b>1.4</b> | <b>Omega-3 Polyunsaturated Fatty Acids</b>   |    |
|            | <i>1.4.1. Fatty acid synthesis</i> .....   | 33 |
|            | <i>1.4.2 Dietary Sources of Polyunsaturates</i> .....  | 35 |
|            | <b>1.4.3 Cardioprotective Effects of Omega-3 PUFA</b> .....  | 37 |
|            | <i>1.4.3.1 Absorption of dietary omega-3 PUFA</i> .....  | 38 |
|            | <i>1.4.3.2 Anti-inflammatory effects</i> .....   | 39 |
|            | <i>1.4.3.3 Anti-arrhythmic effects</i> .....   | 40 |
|            | <i>1.4.3.4 Anti-thrombotic effects</i> .....   | 40 |
|            | <i>1.4.3.5 Other cardiovascular benefits</i> .....   | 41 |
| <b>1.5</b> | <b>Effect of Omega-3 PUFA on Anthracycline-induced<br/>Cardiomyopathy - Current Perspectives</b> ..... | 41 |

|            |  |    |
|------------|--|----|
| <b>1.6</b> | <b>Ovine Model of DOX-induced Cardiomyopathy</b> .....                               | 44 |
| <b>1.7</b> | <b>Effects of Omega-3 PUFA in an Ovine Model of DOX-induced Cardiomyopathy</b> ..... | 45 |
|            | <i>1.7.1 Thesis Studies Proposal</i> .....   | 45 |
|            | <i>1.7.1. Study Hypotheses</i> .....   | 46 |

## **Materials and Methods**

|            |   |    |
|------------|---|----|
|            | <i>Animal Ethics Approval</i> .....   | 48 |
|            | <i>Use of Animals and Study Management</i> .....  | 48 |
| <b>2.1</b> | <b>Omega-3 PUFA Dosing Study</b>  |    |
|            | <i>2.1.1 Drenching Protocol</i> .....   | 49 |
|            | <i>2.1.2 Collection of samples for fatty acid level assessment</i> .....  | 49 |
|            | <i>2.1.2.1 Myocardial sample preparation</i> .....  | 50 |
|            | <i>2.1.2.2 Blood sample preparation</i> .....   | 50 |
|            | <i>2.1.2.3 Separation of phospholipids, preparation of fatty acid methyl esters (FAMES) and identification by gas chromatograph</i> ..... | 50 |
| <b>2.2</b> | <b>Ovine Model of DOX-induced Cardiomyopathy</b>  |    |
|            | <i>2.2.1 General Anaesthesia</i> .....  | 51 |
|            | <i>2.2.2 Pericardial Windows</i> .....  | 52 |

|   |    |
|---|----|
| <b>2.2.3 Cardiac Magnetic Resonance Imaging</b>               | 52 |
| 2.2.3.1 Measurement of Left Ventricular Ejection Fraction     | 53 |
| <b>2.2.4 Transthoracic Echocardiogram</b>                     | 54 |
| <b>2.2.5 Blood Samples</b>                                    | 54 |
| <b>2.2.6 DOX-Infusion Protocol</b>                            |    |
| 2.2.6.1 Establishment of dosage                               | 54 |
| 2.2.6.2 Group allocation                                      | 55 |
| 2.2.6.3 Catheterisation and DOX-infusion                      | 55 |
| <b>2.2.7 Retrieval</b>  | 56 |
| <b>2.2.8 Histopathology Protocol</b>                          | 57 |
| <b>2.2.9 Histological Assessment of Percent Area Fibrosis</b> | 57 |
| <b>2.2.10 Sample size calculation</b>                         | 58 |
| <b>2.2.11 Statistical analysis</b>                            | 58 |

## Results

### 3.1 Omega-3 PUFA Dosing Study

#### 3.1.1 Omega-3 PUFA Levels

|   |    |
|---|----|
| 3.1.1.1 Omega-3 PUFA Baseline levels  | 64 |
| 3.1.1.2 Omega-3 PUFA Drenching study - erythrocyte<br>membrane bound levels | 66 |
| 3.1.1.3 Omega-3 PUFA Drenching study-Myocardial levels                      | 66 |
| 3.1.1.4 Myocardial arachidonic acid levels                                  | 68 |

### 3.2 Ovine DOX-infusion Study

#### 3.2.1 Clinical Results

|                        |    |
|------------------------|----|
| 3.2.1.1 Mortality rate | 69 |
|------------------------|----|

|   |    |
|---|----|
| 3.2.1.2 <i>Electrocardiographic changes</i> .....   | 70 |
| 3.2.2 <i>Left Ventricular Ejection Fraction and Volume Changes as Assessed by CMR</i> ..... | 71 |
| 3.2.3 <i>Fractional Shortening as assessed by TTE</i> .....                                 | 75 |
| 3.2.4 <i>Blood Results</i>  |    |
| 3.2.4.1 <i>Troponin-T post DOX infusion</i> .....   | 77 |
| 3.2.4.2 <i>Haemoglobin, WCC, platelets</i> .....  | 77 |
| 3.2.5 <i>Histopathological Assessment</i>   |    |
| 3.2.5.1 <i>Macroscopic Appearances</i> .....  | 80 |
| 3.2.5.2 <i>Histopathological Findings</i> .....   | 82 |
| 3.2.5.3 <i>Ventricular fibrosis burden</i> .....  | 82 |

## **Discussion**

|  |    |
|--|----|
| 4.1 <b>Study Objectives</b> .....  | 86 |
| 4.2 <b>Uptake of Omega-3 PUFA in Merino Sheep</b> .....  | 86 |
| 4.2.1 <i>Elevated Omega-3 PUFA levels at baseline</i> .....                                    | 87 |
| 4.2.2 <i>Implementation of Olive Oil placebo drenching for main study</i> .....                | 88 |
| 4.3 <b>Cardiac effect of Omega-3 PUFA in Ovine model of DOX-induced NICM</b> .....             | 88 |
| 4.3.1 <i>Possible mechanisms for adverse effects of Omega-3 PUFA on DOX-induced NICM</i> ..... | 89 |
| 4.4 <b>Attrition Rate</b> .....  | 90 |

## **4.5 Study Limitations**

|   |     |
|---|-----|
| <i>4.5.1 Anthracycline Administration and Dosage</i> .....                                  | 91  |
| <i>4.5.2 Absence of Neoplasia</i> .....   | 92  |
| <i>4.5.3 General Anaesthesia</i> .....  | 92  |
| <i>4.5.4 Follow up period</i> .....   | 92  |
| <i>4.5.5 Non reporting of some omega-3 PUFA levels and<br/>histopathology samples</i> ..... | 93  |
| <b>Summary and Future Directions</b> .....  | 94  |
| <b>References</b> .....   | 95  |
| <b>Appendix</b> .....   | 113 |

## Abbreviations

|                    |                                    |                |   |
|--------------------|------------------------------------|----------------|---|
| AEC                | Animal Ethics Committee            | EDD            | End-diastolic dimension                         |
| ALA                | Alpha-linolenic acid               | EDV            | End-diastolic volume                            |
| ANOVA              | Analysis of Variance               | EPA            | Eicosapentaenoic acid                           |
| ANP                | Atrial natriuretic peptide         | EPO            | Erythropoietin                                  |
| ARA                | Arachidonic acid                   | ESV            | End-systolic volume                             |
| BSA                | Body surface area                  | ETE            | Eicosatrienoic acid                             |
| CAM                | Cell adhesion molecule             | FOV            | Field of View                                   |
| CH <sub>3</sub>    | Methyl group                       | Fr             | French  |
| CH <sub>3</sub> CO | Acetyl group                       | FS             | Fractional shortening                           |
| CHF                | Congestive heart failure           | G-CSF          | Granulocyte colony stimulating factors          |
| CI                 | Confidence interval                | IC             | Intracoronary                                   |
| CK                 | Creatine Kinase                    | IV             | Intravenous                                     |
| CMR                | Cardiac magnetic resonance imaging | LA             | Left atrium                                     |
| COOH               | Carboxyl group                     | LA             | Linoleic acid                                   |
| COX-2              | Cyclooxygenase-2                   | LARIF          | Large Animal Research & Imaging Facility, IMVS. |
| DGLA               | dihomo-gamma-linolenic acid        | LDH            | Lactate dehydrogenase                           |
| DHA                | Docosahexaenoic acid               | LV             | Left ventricle                                  |
| DNA                | Deoxyribonucleic acid              | LVEF           | Left ventricular ejection fraction              |
| DNR                | Doxorubicin                        | LVEDD          | Left ventricular end-diastolic dimension        |
| DOX                | Doxorubicin                        | LVESD          | Left ventricular end-systolic dimension         |
| DPA                | Docosapentaenoic acid              | m <sup>2</sup> | Metre squared                                   |
| ECG                | Electrocardiogram                  | NADH           | Nicotinamide adenine dinucleotide hydrogenase   |

### Abbreviations (continued)

|      |                                 |       |                                    |
|------|---------------------------------|-------|------------------------------------|
| NICM | Nonischaemic<br>Cardiomyopathy  | SD    | Standard Deviation                 |
| PG   | Prostaglandin                   | SR    | Sarcoplasmic reticulum             |
| PLA2 | Phospholipase A2                | TE    | Echo Time                          |
| PUFA | Polyunsaturated fatty acid      | TPO   | Thrombopoietin                     |
| RA   | Right Atrium                    | TR    | Repetition Time                    |
| RBC  | Erythrocyte (red blood<br>cell) | TTE   | Transthoracic<br>Echocardiography  |
| ROS  | Reactive oxygen species         | VA    | Ventricular Arrhythmia             |
| RV   | Right ventricle                 | V-CAM | Vascular cell adhesion<br>molecule |
| SEM  | Standard Error of the<br>Mean   | WCC   | White Cell Count                   |