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

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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 affects, 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.

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Angelo Carbone, BSc.
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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 naturetic 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_3	Methyl group	Fr	French
CH_3CO	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 c acid	LV	Left ventricle
DNA	Deoxyribonucleic acid	LVEF	Left ventricular ejection fraction
DNR	Duanorubicin	LVEDD	Left ventricular end-diastolic dimension
DOX	Doxorubicin	LVESD	Left ventricular end-systolic dimension
DPA	Docosapentaenoic acid	m^2	Metre squared
ECG	Electrocardiogram	NADH	Nicotinamide adenine dinucleotide hydrogenase

Abbreviations (continued)

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

Introduction

1.1 Cancer and Chemotherapy

1.1.1 Cancer

Cancer is a disease typically characterised by changes in deoxyribonucleic acid (DNA) leading to the uncontrolled division of abnormal cells. It remains one of the leading causes of death in all Western industrialised countries[1-6]. Over 93,000 new cases of cancer were reported in Australia in 2003 and there is a one in two and one in three chance of a cancer diagnosis before age 85 in males and females respectively[7].

1.1.1.1 Current treatment options

Current evidence-based treatment options for cancer include surgery, radiotherapy and chemotherapy. Chemotherapy agents are typically administered intravenously at regular intervals over weeks or months. Until recently, chemotherapy was more commonly indicated in advanced cancer stages (III or IV), but its use is now expanding to earlier stages of disease progression[8]. Its administration is often divided into three stages, *induction*, where the goal is to reduce the number of cancer cells, *consolidation*, to achieve complete remission, and finally, *maintenance*, given after remission to prevent relapse of the disease[9]. It has advantages over surgery and radiotherapy in that delivery of the chemical agent is via the body's own blood stream and can therefore be particularly effective against

cancers not localised to a specific body area or those areas difficult to access surgically.

1.1.2 Anthracyclines

Doxorubicin (Adriamycin) (DOX), daunorubicin (DNR) and epirubicin are bacterial antibiotics widely used as chemotherapy agents in the treatment of various forms of cancer. They are classed as anthracyclic glucosides (anthracyclines) in that they target particular parts of the DNA chain and work by impairing DNA replication in rapidly dividing cells, such as malignant cancer cells. They are administered as single agents or in combination with systemic adjuvants or other adjunctive agents. They are isolated from a soil based pigment producing bacterium *Streptomyces peucetius,* and have been used extensively since the late 1960's because of their powerful anti-tumour effects against several types of cancer. This includes acute leukaemia, Hodgkin's and non-Hodgkin's lymphoma, Kaposi's sarcoma, soft tissue sarcomas, osteosarcomas, carcinomas, and breast cancer, all of which can occur in young patients and have some potential for cure[10, 11]. Almost 60% of children diagnosed with cancer receive anthracyclines as part of their treatment[12]. Doxorubicin is also referred to as an anthracycline quinone, because of the 1,4 benzoquinone ring ($C_6H_4O_2$) which is an integral part of its structure and properties **(Figure 1.1.1).**

1.1.3 Cytotoxic Effects of Doxorubicin

Chemotherapy agents by nature are cytotoxic. A common side effect of anti-tumour agents such as DOX is that they also attack healthy cells that are dividing normally, such as hair follicle, bone marrow and intestinal cells. This is associated with many of the common adverse effects attributed to chemotherapy such as nausea, alopecia,

skin lesions, diarrhoea, nervous system and other organ damage, fatigue, anxiety and depression[9]. There are several mechanisms proposed for the cytotoxicity of DOX.

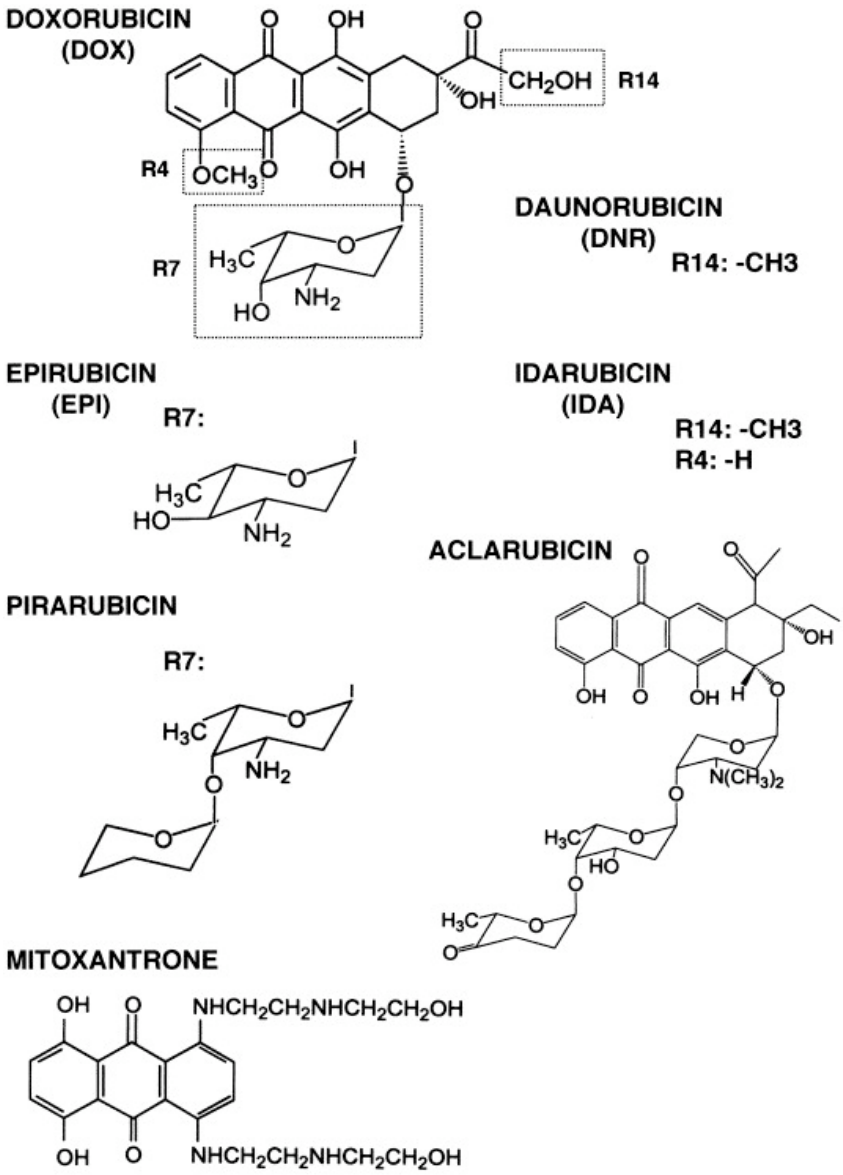


Figure 1.1.1 Chemical structures of well known anthracyclines. The quinone ring ($C_6H_4O_2$) is the second carbon ring from left. (Image reprinted from Takemura et al 2007[10])

1.1.3.1 Generation of reactive oxygen species (ROS)

The quinone ring within DOX is prone to liberation of oxygen free radicals[13]. Once administered, DOX is rapidly absorbed into cells and accumulates in microsomes[14, 15], sarcosomes[15] and mitochondria where it can reach concentrations two orders of magnitude higher than in the extracellular fluid[16, 17]. It is then metabolised by mitochondrial enzymes, and in particular, nicotinamide adenine dinucleotide (NADH) [18, 19]. The action of NADH on DOX causes redox cycling of the quinone ring between quinone and semi-quinone states **(Figure 1.1.2).** The quinone ring is reduced to semiquinone by addition of one electron in a process catalysed by NADH[10, 18]. This process causes release of electrons from the quinone ring which are captured by oxidizing agents, including oxygen, which then initiate a chain reaction leading to generation of superoxide and other ROS[10,18]. This causes non-specific oxidation of cellular structures including cell membrane phospholipids, cellular organelles and nucleic acids. Doxorubicin also causes a reduction in the levels of endogenous antioxidants which scavenge free radicals, further exacerbating its oxidative effects[20, 21].

Finally, DOX readily binds endogenous iron, forming complexes which have the dual properties of preferentially binding to DNA, whilst simultaneously releasing hydroxyl radicals which can break DNA chains[22-25].

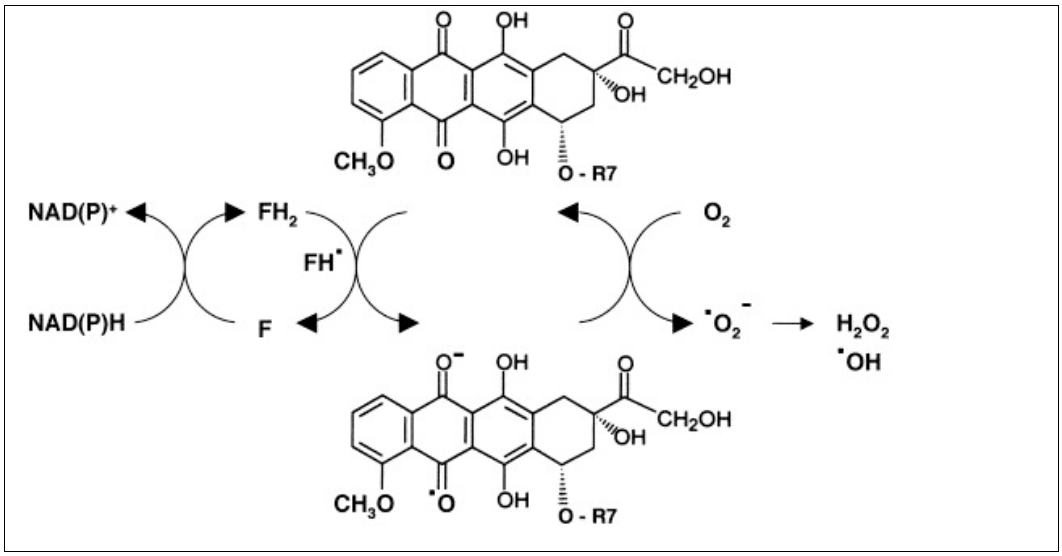


Figure 1.1.2 Reactive oxygen species formation through reductive activation of DOX. FH2 indicates reductases or dehydrogenases. FH indicates flavin semiquinone. (Image reprinted from Takemura et al 2007[10]).

1.1.3.2 Antioxidant adjuvants to DOX therapy.

Studies have shown that antioxidant agents including vitamin C[26, 27], superoxide dismutase[26], N-acetylcysteine[28], glutathione[28, 29], catalase[26], and probucol[30] reduced the oxidative stress caused by DOX, without diminishing the cytotoxic effects on tumour cells. This suggests that DOX' anti-neoplastic effects are not mediated predominantly due to oxidative damage of nucleic acid molecules and raises the prospect that antioxidants may be used as clinical adjuncts to selectively protect against antihracycline toxicity without compromising therapeutic benefit.

1.1.3.3 Mechanisms of anthracycline-induced anti-tumour activity

There are several mechanisms proposed for the specific anti-tumour activity of anthracyclines. These relate principally to the prevention of replication of rapidly dividing cells.

Metabolites of DOX covalently bind with high affinity to DNA bases[31, 32]. This interferes with the unlinking of DNA base pairs by helicases[32], a process crucial to DNA replication. It also inhibits synthesis of a new DNA strand by inhibition of DNA polymerase. A single molecule of DOX is sufficient to irreversibly block helicase, an enzyme that disrupts the hydrogen bonds that stabilise and hold the two complementary DNA strands together[33]. DOX also inhibits topoisomerase II which is a critical enzyme in the separation of replicated sister chromatids following DNA replication[34-36, 37] This occurs via formation of a DOX topiosomerase II-DNA complex[37]. Furthermore, this complex prevents repair of broken DNA strands[38-40]. DOX has also been shown to inhibit DNA ligase activity which is crucial in the joining of DNA strands by a phosphate ester bridge[38].

1.1.3.4 Apoptosis

Apoptosis, also termed "programmed cell death", plays a central role in many normal biological processes, such as morphogenesis, cell turn over, hormone-dependent organ atrophy, and immune system function. It is as important to the growth and development of normal tissue as mitosis. Apoptosis comprises a well-described cascade-like process that results in progressive changes to cellular components including nucleic acid fragmentation, chromatin condensation and cytoskeleton degradation[41 42].

Most anticancer drugs induce apoptosis[43], with this process affecting normal as well as tumour cells. It has been suggested that alopecia during chemotherapy is the result of induced apoptosis of hair follicle cells[44]. However, the precise mechanisms of chemotherapy-induced apoptosis are still contentious[43].

Caspases are a family of cysteine proteases that play essential roles in the apoptosis signalling pathway. Activated caspases cleave intercellular proteins in a cascade-like manner leading to release of cytochrome c from mitochondrial lamellae. This process stimulates further enzymatic activation of caspases and results in cleavage of the actin cytoskeleton, cell condensation and cell death. Ceramides are a family of lipid molecules concentrated in cell membranes. They play a crucial role in the signalling pathway of anthracycline-induced apoptosis by recruitment and activation of caspases following internalisation of DOX. Apoptosis also involves fragmentation of DNA by endonucleases[45], which in turn contributes to the well-characterised phenomenon of chromatin condensation. Although apoptosis does not lead to cell lysis, inflammatory response, or necrosis (which are features typical of uncontrolled cell death and which further damage adjacent cells), unregulated apoptosis can be equally devastating to normal tissue structure and function and potentially lead to adverse health effects.

1.2 Doxorubicin-induced Cardiomyopathy

That DOX and other anthracyclines are very potent, effective and widely used anti-tumour agents, albeit with many of the typical adverse effects attributed to chemotherapy, is well established. *However its major clinical shortcoming is that it may cause dose-dependent, non-ischaemic dilated cardiomyopathy (NICM) that can lead to congestive heart failure (CHF)*. This dose- dependent association between DOX and onset of cardiomyopathy leading to CHF was first reported by Lefrak et al in 1973[46]. The authors described a retrospective review of 399 subjects. The incidence of DOX-induced CHF was more than 4% of patients who received a cumulative dose of 500-550mg/m^2 body surface area (BSA) rising to 18% and 36%

for cumulative doses of 550-600mg/m^2 and greater than 601mg/m^2 respectively. Patients older than 60 years are at greater risk with a 4.6% incidence reported at only 400mg/m2 DOX cumulative dose[47]. The prognosis for DOX-induced CHF has historically been reported as very poor, with up to 20% mortality reported in an early series[48]. In a series of 201 young patients, a 23% incidence of cardiac abnormalities presenting four to 20 years after anthracycline therapy was reported[49]. These patients had received a median anthracycline dose of 450mg/m^2 BSA. Dose-dependent anthracycline-induced cardiotoxicity has also been reported in other large patient cohort clinical studies[50, 51]. The paediatric population are particularly vulnerable to the cardiotoxic effects of DOX and other anthracyclines[50, 52, 53], with one series reporting five deaths from a total six cases[53]. Some studies suggest that enhanced cardiotoxicty may develop at doses lower than 400mg/m^2 BSA where multi-anti tumour agent therapy is administered[51].

The study by Lefrak concluded that the total cumulative dose of DOX should not exceed 550mg/m^2 of body surface area (BSA) [46]. Seventy five mg/m^2 represents a typical single dose range for DOX chemotherapy[54-56]. Therefore the safe threshold for cardiac complications is exceeded with relatively few doses. In addition, the cumulative dose refers to that received over a lifetime[57], such that repeat courses of DOX chemotherapy following relapse are likely to be problematic.

Cardiotoxicity is the single most common factor that limits anthracycline use clinically and this is particularly so for patients with risk factors for increased toxicity, including those who have received anthracyclines in the past, older patients, or those who are also to receive other cardiotoxic agents[58].

1.2.1 Mechanisms of Anthracycline Induced Cardiomyopathy

1.2.1.1 Apoptosis and oxidative stress

Many of the adverse cellular effects of DOX described in the preceding sections, including non-specific oxidative damage and apoptosis, also relate significantly to the myocardium[18, 19, 59]. Redox recycling of anthracyclines by mitochondrial NADH generates ROS including O_2-, H_2O_2 and OH and which may underlie their cardiotoxicity[18]. In addition, exposure of endothelial cells and cardiomyocytes to DOX causes apoptosis at sub-micromolar concentrations[59]. Increased oxidative stress may lead to a variety of changes to cardiomyocyte subcellular structures including loss of myofibrils and cytoplasmic vacuolization[11] **(Figure 1.2.1)** with the end result being apoptosis and replacement fibrosis of affected cells[10] **(Figure 1.2.2)** .

Cardiac muscle oxidative injury associated with anthracycline use is marked by several other sub-cellular abnormalities, including distortion and disruption of mitochondrial and sarcoplasmic reticulum (SR) membranes[46]. The myocardium contains particularly high mitochondrial density to enable its continuous aerobic respiration during cyclic contraction[20], and is poorly endowed with antioxidant defence mechanisms[60]. These factors may account for its particular susceptibility to oxidating agents. However, there are a number of other mechanisms proposed relating specifically to myocardial susceptibility to anthracyclines.

1.2.1.2 Down -regulation of cardiac specific muscle proteins

DOX down-regulates the expression of a variety of cardiac muscle-specific proteins, most notably α-actinin[61]. This is an essential component of the contractile apparatus in cardiomyocytes. Reduction in myoprotein expression is associated with the reduced contractility of myocardium and may explain the pathological features of

myofibrillar loss in DOX induced cardiomyopathy[10] as well as the decreased contractility. Doxorubicin up-regulates expression of atrial naturetic peptide (ANP), a polypeptide hormone involved in the homeostasis of atrial myocytes. This up-regulation can persist constitutively, even following cessation of chemotherapy, and has been proposed as a factor in those cases of cardiomyopathy presenting months or years following cessation of therapy[62].

1.2.1.3 Release of vasoactive substances

Administration of DOX has also been shown in a canine model to cause profound, acute, haemodynamic changes involving release of histamine[63]. This was followed by the secondary release of catecholamine, notably adrenaline, which caused an increase in levels of immunoreactive prostaglandins (PG) in the coronary sinus[63]. Such induction in the release of vasoactive substances by anthracyclines could be another pathogenic factor in the onset of cardiomyopathy.

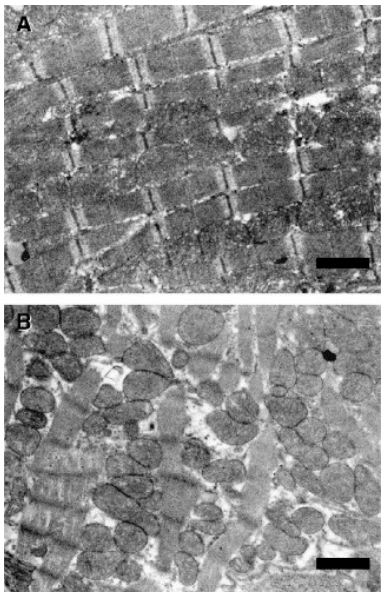


Figure 1.2.1 Electron micrograph of normal (upper) and DOX-treated mouse heart (lower), showing loss of myofibrils in the DOX-treated cardiomyocytes. Bars 1 *u*m.

Figure 1.2.2 Masson's trichrome stain (upper), and Haemotoxylin-eosin (middle and lower) of DOX-treated cardiomyocytes showing extensive replacement fibrosis (upper) vacuolar degeneration (middle) and foci of necrotic cardiomyocytes (lower). Bars 20*u*m. (Figures 1.2.1 and 1.2.2 reprinted from Takemura et al 2007[10]).

1.2.2 Cardiac Monitoring of Patients Receiving Anthracyclines

The consequences of heart damage caused by anthracyclines are extensive. Cardiotoxicity can cause severe morbidity and lead to a reduction in quality of life. As discussed, it limits further administration of the drug, potentially affecting chances of patient survival. It requires long term treatment, incurring high medical costs, and causes premature death[12].

Intensive cardiac monitoring including echo and electro- cardiography (ECG) is routinely conducted during chemotherapy. Further follow-up evaluation may be indicated for many years following cessation of anthracycline administration[49, 64]. Electrocardiogram (ECG) changes associated with DOX-induced cardiomyopathy include various arrhythmias, most commonly sinus tachycardia (abnormally rapid heart rate), and anomalies to the ECG wave morphology. Echocardiography, often combined with exercise stress testing, is used to monitor anatomical and functional changes including changes to ventricular ejection volumes. Radionuclide cardiac scintigraphy, utilizing radio-labelled dye injected into the left ventricle (LV) is also used to measure global ejection fraction, and detect regional wall contractile loss and myocarditis. However, pathognomic features of anthracycline induced cardiomyopathy in chemotherapy patients are difficult to diagnose noninvasively[11], and positive findings from these investigations indicate that the disease process is already established. The greatest specificity and sensitivity for anthracycline-induced cardiomyopathy is provided by endomyocardial biopsy[65], which is used to demonstrate histopathological changes described earlier, including myofibril loss, distortion and disruption of mitochondrial and SR membranes and cytoplasmic vacuolization. However this procedure is particularly invasive, expensive and available in few centres [66].

1.3 Treatment Strategies to Reduce Anthracycline-induced NICM

A number of strategies have been devised and evaluated to reduce the cardiotoxic effect of anthracyclines. These include modifying dosing strategies, the use of novel anthracycline derivatives, and co-administration of adjunctive agents.

1.3.1.1 Dosing regime

Different DOX dosing strategies have been compared for their effect on cardiotoxicity, including continual 48-96 hour infusion and standard bolus intravenous injection [67, 68]. Constant infusion has been associated with a significant reduction in the number of patients showing severe morphologic changes in myocardial biopsy specimens[67], and an accompanying reduction in the severity of systemic effects such as nausea and vomiting[68].

However, a recent meta-analysis review of five European randomised prospective clinical trials of anthracycline-induced cardiotoxicty in children, found insufficient evidence that changing the dosing infusion strategy led to reduced cardiotoxicity in these patients[12].

1.3.1.2 Anthracycline analogues

5-iminodaunodrubicin is an anthracycline analogue which has been shown in a bovine heart sub-mitochondrial model to have diminished cardiotoxic potential due to having little or no tendency to undergo oxidation[18]. To date this has not been confirmed in clinical studies. In an overview of preclinical and clinical studies, 4'-epi-doxorubicin, another analogue of DOX, showed qualitatively similar, but quantitatively less acute cardiac toxicity compared to identical doses of DOX[69]. However this may have been at the expense of altered anti-tumour activity[69].

1.3.1.3 Liposomal preparations

Other clinical and pre-clinical studies have shown that encapsulating conventional anthracyclines in liposomes (cell membrane vesicles) reduces the incidence and severity of cumulative dose-related cardiomyopathy while preserving anti-tumour activity[58]. However, at present these liposomal equivalents are prohibitively expensive for routine use compared with conventionally prepared agents.

1.3.2 *Cardioprotective Adjuncts*

1.3.2.1 Dexrazoxane

As described earlier, DOX readily binds endogenous iron and the resulting complexes preferentially bind DNA whilst simultaneously releasing hydroxy radicals which can cause oxidative stress to DNA and adjacent cellular structures. Dexrazoxane is an iron chelator which has been shown in a canine model to reduce DOX-induced cardiotoxicity whilst not appreciably limiting its anti-neoplastic activity[70]. This has been demonstrated in a study of 101 DOX-treated children with acute lymphoblastic leukemia[71] and in several other clinical trials[72]. Although dexrazoxane can increase tolerance to DOX in terms of dose-dependent cardiomyopathy[72], its benefit to long term survival remains unclear[73]. Adverse effects associated with the use of dexrazoxane in patients receiving DOX include increased myelosuppression, infection and fever[74]. Meta-analysis studies have also indicated that dexrazoxane may reduce the anti-tumour efficacy of the drug[12].

1.3.2.2 Hematopoietic cytokines

More recently, haematopoietic cytokines have been investigated as adjunctive agents to anthracyclines for their ability to decrease cardiomyopathy and/or other

adverse effects. These include granulocyte-colony stimulating factors (G-CSF) and thrombopoietin (TPO). Granulocyte-colony stimulating factor has been used to reduce the incidence of granulocytopaenia and infections in elderly patients receiving DOX[75]. Thrombopoietin has been shown in a small animal study to protect against DOX-induced vacuolization, myofibrillar loss and apoptosis in spontaneously beating cells of primary neonatal rat ventricle, whilst increasing heart rate, ventricular function and output[57]. Clinical studies to investigate supplementation of DOX with G-CSF and TPO in patients with sarcomas are underway[56] but results have not yet been reported. Erythropoietin (EPO) has also been studied for its cardioprotective effects. This agent appeared to provide cardiomyocyte protection against acute DOX toxicity in a rat organ bath model[76] and in experiments with cultured rat cardiomyocytes[77]. However, demonstration of a clinical benefit is once again lacking.

1.3.2.3 Antioxidants

Although antioxidants such as Vitamin E and N-acetylcysteine are prime candidates for evaluation against anthracycline-induced cardiomyopathy, they have not been shown to prevent nor delay the onset of cardiomyopathy in clinical studies[78, 79].

1.3.2.4 Improved prognosis with current generation heart failure medications

A recent clinical study reported a significant improvement in left ventricular ejection fraction (LVEF) in 42% of 201 cases of anthracycline-induced NICM receiving current generation medications (enalapril and carvedilol) for the treatment of CHF[80]. This compares favourably to earlier reported series using contemporary

HF medications. The study concluded that LVEF recovery may be achieved when cardiac dysfunction is detected early and a modern HF treatment is initiated promptly. However, these agents achieve improvement in LVEF by modulating the renin-angiotensin hormone systems that regulate blood pressure and fluid balance, rather than via attenuation of cardiomyopathy onset itself. There remains considerable scope for further enhancement in the prevention and management of anthracycline-induced NICM.

To date, the evidence for most of the candidate agents has been restricted to *in vitro* and small animal studies and results from the small number of clinical studies have been disappointing. The emergence of a safe, effective, tolerable and affordable adjunctive therapy which could attenuate the onset of anthracycline cardiotoxicity would represent a significant advancement in the management of neoplastic disease.

1.4 Omega-3 Polyunsaturated Fatty Acids

Fatty acids are an important source of energy, are integral to many protein structures and are an essential component of cell membranes. They comprise molecular chains of between two and 24 carbon atoms with a carboxyl group (COOH) at the terminus, also known as the “delta” end, and a methyl (CH_3) at the opposite, also termed “omega” end. They are partly classified according to the level of saturation of the carbon atoms within the chain by hydrogen, (ie whether each carbon has the maximum number of hydrogen atoms attached). A saturated fatty acid indicates that all of the available bonds to the carbon atoms within the chain are occupied by hydrogen **(Figure 1.4.1)**. An unsaturated fatty acid describes a fatty acid chain in which there are one or more carbon-carbon double bonds and therefore one or more

points capable of supporting additional hydrogen atoms that are not currently part of its structure.

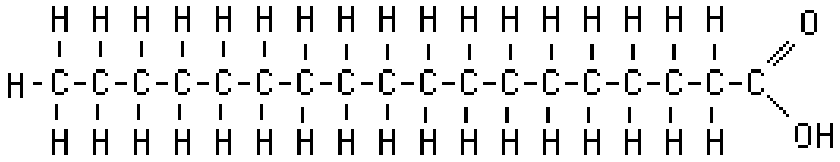


Figure 1.4.1 Stearic acid, an 18 carbon chain saturated fatty acid.

Unsaturated fatty acids comprise mainly 18, 20 or 22 carbon atoms in the chain and are denoted by the formula “a:bn-c” where “a” is the number of carbon atoms in the chain, “b” the number of carbon-carbon double bonds, and “c”, the position of the first double bond in the chain relative to the omega (methyl) end. For example, linoleic acid (LA), an omega-6 polyunsaturated fatty acid (PUFA) derived from plants, is denoted as 18:2n-6, where “18” indicates the number of carbon atoms in the chain, “2” indicates two carbon-carbon double bonds and “6” indicates that the first of the double bonds occurs between the 6^{th} and 7^{th} carbon from the methyl (or omega) end. The terms “omega” and “n-“ are inter-changeable.

1.4.1 Fatty acid synthesis

Fatty acids can be synthesised *de novo* in all organisms. Acetyl-CoA, a dimeric molecule consisting of an acetyl (CH_3CO) group attached to coenzyme A is the precursor molecule. Coenzyme A can perform dual roles, including oxidation of fatty acids to generate energy via Krebs citric acid cycle and fatty acid synthesis. In humans, most fatty acid synthesis occurs in the liver and adipose tissues. Fatty acid synthase, a large dimeric protein, catalyses the elongation of fatty acid chains from the acetyl-Co A precursor.

Stearic acid is an 18 carbon saturated fatty acid (18:0), synthesised by all organisms. It is the major precursor for PUFA, which are formed by the insertion of carbon-carbon double bonds by desaturase enzymes. Desaturase enzymes are named from the first carbon atom from the delta (carboxyl) end of the molecule which has a double bond. Hence stearic acid (18:0) is converted to oleic acid (18:1n-9) by Δ-9 desaturase, as it catalyses the insertion of a double bond to the 9th carbon atom in the chain from the delta end. **(Figure 1.4.2)**. Oleic acid is further converted by Δ-12 desaturase to LA (18:2n-6), as it now has two carbon to carbon double bonds, with the first located at the 6th carbon from the omega end.

Further desaturation by Δ-15 desaturase converts LA to α-linolenic acid (ALA, 18:3n-3), now with the first double bond located at the 3rd carbon from the omega end. Animals do not possess either the Δ-12 desaturase or the Δ-15 desaturase enzymes. Therefore, both the omega-6 and omega-3 PUFAs are classified as essential fatty acids as they must be obtained from dietary sources. Both LA and ALA can undergo further desaturation and elongation (ie addition of acetyl group to alpha-end), as depicted in **Figure 1.4.2**.

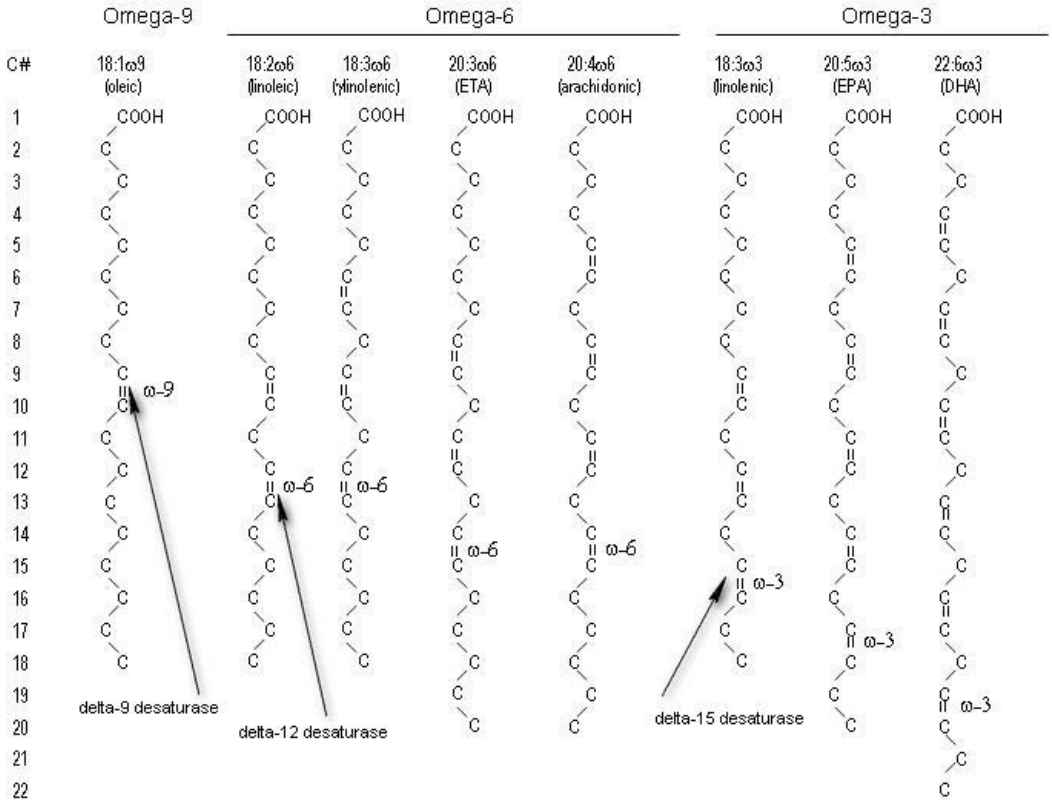


Figure 1.4.2 Synthesis of n-9, n-6 and n-3 fatty acids. Oleic acid, an n-9 fatty acid is converted to linoleic acid, an n-6 fatty acid by Δ-12desaturase, which is in turn converted to linolenic acid, an n-3 fatty acid by Δ-15 desaturase.

1.4.2 Dietary Sources of Polyunsaturates

The relative proportions and total intake of dietary fats changed significantly during the evolutionary stages of human society **(Figure 1.4.3)**. The Western diet is abundant in omega-6 PUFA, mainly from plant oils. In Australia, the most common sources are sunflower, safflower, corn, peanut and cottonseed oils, which are rich in linoleic acid[81]. It is estimated that typical Western diets have an omega-6 : omega-3 PUFA ratio of approximately 10:1[81]. Linoleic acid can be converted to the omega-3 α-linolenic acid in small quantities in plants, these being the major constituents of most plant lipids. However, only marine microorganisms and a small number of terrestrial fungal species have the required enzymes to more efficiently convert the 18 carbon linoleic and α linolenic acids into the longer (greater than 20 carbon chain)

omega-6 and omega-3 PUFA derivatives, arachidonic (ARA) and eicosapentaenoic acid (EPA) respectively **(Figure 1.4.4)**. Arachidonic acid and EPA can be further converted by these organisms to 22 carbon chain docosapentaenoic (DPA) and docosahexaenoic acid (DHA). These fatty acids are assimilated up the marine food chain and ultimately into the seafood products consumed by man.

NOTE:
This figure is included on page 37 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4.3 Hypothetical scheme of the relative percentages of fat and different fatty acid families in human nutrition as extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative changes during the preceding 100 y. *trans* Fatty acids, the result of the hydrogenation process, have increased dramatically in the food supply during this century (reprinted from Simopoulos A. Am J Clin Nutr, 1999;70(3);560S-569S).

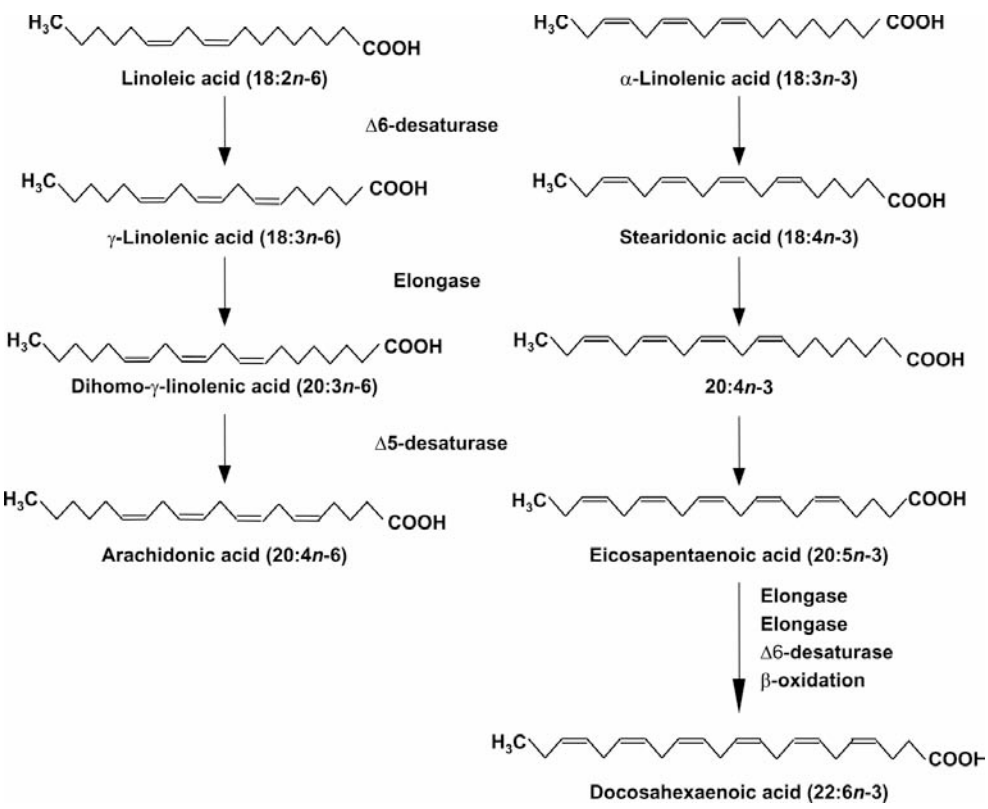


Figure 1.4.4 Conversion of LA and ALA to longer chain metabolites by desaturation and elongation

1.4.3 Cardioprotective Effects of Omega-3 PUFA

Omega-3 PUFA have emerged in recent years as a protective dietary component against coronary heart disease and stroke. This has stemmed from initial observations in the 1970s of significantly lower mortality rates from coronary heart disease in countries such as Greenland and Japan, where seafood comprises a large component of the typical diet. Many recent human population and randomised clinical studies have since reported the role of omega-3 PUFA as a protective dietary component against coronary heart disease and stroke, with the longer chain omega-3 EPA and DHA shown to provide the greatest cardiac benefit[82-86]. There is strong evidence that modest consumption of fish or fish oil (1-2 servings per week of oily deep sea fish (eg tuna, salmon or mackerel), or 250mg/day of EPA+DHA supplement, provides

sufficient levels to reduce the risk of sudden cardiac death by 36%, regardless of presence or absence of established CVD [82].

1.4.3.1 Absorption of dietary omega-3 PUFA

Following ingestion, EPA and DHA are rapidly absorbed through the gut and into the blood plasma. If sufficient intake levels are maintained over several days, they incorporate into cell membrane phospholipid bilayers, where they substitute arachidonic acid (ARA), a 20 carbon chain omega-6 PUFA, in a process involving phospholipase A2 (PLA2) **(Figure 1.4.4)**. Incorporation of PUFAs into membranes is a result of their continual turnover, and membrane levels of PUFAs reflect the relative proportions of PUFAs available. Membrane levels also reflect the degree to which tissues conserve omega-3 PUFA already present, ie, some tissues selectively release omega-6 before omega-3 PUFAs. Incorporation of EPA and DHA causes a decrease in membrane bound ARA. The incorporation of omega-3 PUFA into the membrane phospholipid bilayer is a prerequisite for its biological activity and imparts numerous cellular effects, including changes to membrane polarisation properties, protein activities, matrix remodelling and modulation of gene expression[87].

NOTE:
This figure is included on page 40 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4.4
Incorporation of Omega-3 fatty acids.
Ingestion of omega-3 causes a rapid rise in omega-3 plasma levels. If sufficient ingestion levels are maintained, Omega-3's incorporate into cell membrane phospholipid bilayer by substituting arachidonic acid, an omega-6 fatty acid. Membrane incorporation and maintenance of membrane bound levels of omega-3 are prerequisite for cellular changes in response to omega-3's and impart numerous cellular effects, including modulation of bioactive lipid mediators and signalling molecules, altered membrane protein activity and transduction of signalling pathways within the cell leading to modified gene and ultimately modified protein expression. Image reprinted from Surette, M.E.CMAJ 2008; 178: 177-180.

1.4.3.2 Anti-inflammatory effects

A number of mechanisms have been proposed to describe the cardio-protective effects of omega-3 PUFA. Following dietary supplementation, EPA and DHA have been shown to incorporate into the membranes of human cardiomyocytes[88]. As discussed, incorporation of EPA and DHA into cell membrane phospholipids is via substitution of omega-6 PUFAs of which ARA is the major component. Both ARA and EPA are precursors for eicosanoids, including prostaglandins and thromboxanes

(via the lipoxygenase pathway) and leukotrienes (via the lipoxygenase pathway) and act as signalling control molecules for inflammatory and immune processes. It is widely regarded that eicosanoid derivatives from omega-6 PUFA such as ARA tend to be pro-inflammatory, whilst those derived from omega-3 PUFA are significantly less so[89]. Membrane phospholipid-bound ARA, released by phospolipase A, forms the predominant precursor of pro-inflammatory eicosanoids. Substitution of membrane bound ARA with EPA/DHA therefore reduces the availability of this omega-6 PUFA for conversion to pro-inflammatory eicosanoids, and thereby reduces the response to inflammatory stimuli in tissues, including myocardium.

1.4.3.3 Anti-arrhythmic effects

It is now well established that incorporation of omega-3 PUFA into cardiomyocyte membranes alters their voltage conduction properties such that they demonstrate more controlled sinus rhythm and resistance to anti-arrhythmia[90, 91]. Enhancement of the conduction properties of cardiomyocyte cell membranes following incorporation of EPA and DHA is believed to be a predominant mechanism underlying the cardioprotective properties of omega-3 PUFA[90-92]. The anti-arrhythmic effects of omega-3 PUFA were well characterised in a study by Billman et al[93], in a canine model of inducible ventricular fibrillation (VF), where infusion of omega-3 PUFA in these animals delayed onset of VF in response to exercise stress compared with baseline values.

1.4.3.4 Anti-thrombotic effects

Several studies have demonstrated the anti-thrombotic properties of omega-3 fatty acids. Human consumption of 3-4g/day of EPA/DHA has been shown to reduce

thrombosis by decreasing platelet aggregation induced by collagen and platelet activation factor[94]. Cell adhesion molecules (CAM) are trans-membrane proteins that play several key roles in tissue homeostasis and development. They function by modulating binding of particular cell types. Certain CAMs such as vascular cell adhesion molecules (VCAM-1) also play a role in disease processes such as inflammation and atherogenesis. It is difficult to measure membrane levels of adhesion molecules in the human vasculature following DHA supplementation, however it has been shown in cultured human saphenous vein endothelial cells to reduce membrane surface expression of these molecules[95]. Thromboxane A2 is a pro-thrombotic eicosanoid synthesized in platelets via cyclooxygenase action on its precursor molecule ARA. Omega-3 PUFA reduces production in platelets. These properties are likely to be relevant to the anti-thrombotic properties of omega-PUFA.

1.4.3.5 Other cardiovascular benefits

Other studies have shown a modest but dose-dependent improvement in endothelial function[95], with a resulting decrease in blood pressure[96], and lowering of plasma triglyceride levels[97] in response to dietary omega-3 PUFA supplementation.

1.5 Effect of Omega-3 PUFA on Anthracycline-induced NICM – Current Perspectives

Few studies have investigated the effects of omega-3 PUFA supplementation in the presence of anthracycline agents, with all of these conducted *in vitro* or in small animal neoplastic models. Germain *et al,* reported that dietary supplementation of

omega-3 PUFA increased rat mammary tumour sensitivity to epirubicin without altering its cardiac toxicity[98]. All animal cells, including tumour cells, can potentially incorporate EPA and DHA into their phospholipid membranes during dietary supplementation. In addition, EPA and DHA, due to their lower level of saturation compared with ARA and other membrane phospholipids, may also be more prone to lipid peroxidation. This was proposed as the mechanism underlying the sensitisation of neoplastic cells by omega-3 PUFA in the rat mammary study[98]. Since lipid peroxidation may also mediate anthracycline induced cardiotoxicity, it is important to determine whether omega-3 PUFA supplementation may exacerbate or reduce this serious side effect. A more recent study monitored cardiac function in omega-3 PUFA fed Sprague Dawley rats during co-administration of epirubicin[99]. They showed no additive nor preventative effect of omega-3 PUFA on cardiotoxicity. Dietary fish oil supplementation in mice has also been shown to augment the efficacy of DOX against breast cancer xenografts without increasing non-cardiac drug toxicity[100]. Although these findings are reassuring and raise the potential of omega-3 PUFA as a tumour sensitising agent, conflicting evidence also exists to suggest that dietary omega-3 PUFA may diminish cellular anti-oxidant defences, which in turn may accentuate cardiomyocyte susceptibility to lipid peroxidation after exposure to DOX[101]. Table 1.5.1 summarises the outcomes of several small animal and *in vitro* models investigating the effects of omega-3 PUFA supplementation with co-administered anthracyclines.

As discussed, all published findings to date are confined to small animal or *in vitro* neoplasia models with many not assessing cardiac function directly. Further, some results indicate that dietary supplementation of omega-3 PUFA in association with anthracycline administration may exacerbate cardiotoxicity. Clearly there is a need

to evaluate the cardio-protective effects of omega-3 PUFA in a large DOX treated *in vivo* model comparable to human cardiovascular anatomy and physiology, before a clinical trial evaluating its safety and effectiveness in patients receiving anthracyclines can be considered.

Detrimental Effect	**No Change or Indeterminant**	**Benefit**
Matsui et al, CanJCard [101]. Rat DOX model ± omega-3 PUFA	*Germain et al, Pharm Res*[99]. Rat epirubicin model ± omega-3 PUFA	*Germain et al, Lipids*[98]. Rat mammary tumor model + epirubicin ± DHA
	Selting et al, Am J Vet Res[102]. Dog lymphoma study + DOX + Hi/Low dose omega-3 PUFA	*Colas et al, Clin Canc Res*[103]. Rat mammary tumor model + epirubicin ± DHA
	Rudra et al, Anti Canc Res[104]. Cancer Cell lines + DOX ± DHA	*Hardman et al, Clin Canc Res*[100]. Mouse cancer xenograft model + DOX ± omega-3 PUFA

Table 1.5.1. Summary of conclusions from several small animal and in vitro studies investigating the effects of omega-3 PUFA on anthracycline induced cytotoxicity. There is no clear consensus regarding it's benefit or otherwise.

1.6 Ovine Model of DOX-induced Cardiomyopathy

Previous work undertaken by this department has validated a sheep model of DOX-induced, non-ischaemic cardiomyopathy (NICM)[105]. In this study 10 healthy Merino wethers received fortnightly intra-coronary (IC) infusions of DOX for a mean cumulative dose of 3.6mg/kg. Dosing was targeted to the coronary arteries to minimise systemic adverse effects (e.g. myelosuppression, diarrhoea, anorexia) and to cause reproducible cardiotoxicity. The development of toxic cardiomyopathy was validated by cardiac magnetic resonance imaging (CMR), including late uptake of gadolinium to assess for scar tissue formation. Magnetic resonance imaging is the current "gold standard" for non-invasive assessment of cardiac volumes and function[106] and enabled high resolution assessment of the myocardium. Additional assessment was performed with transthoracic echocardiography, haemodynamic measurements and histology. The doxorubicin dosing regimen was generally well tolerated with systemic toxicity consisting mainly of reversible myelosuppression. Premature attrition rate was 20%, with both deaths due to cardiac arrhythmia, but which was a substantial improvement compared to previous studies with reported mortality rates of up to 60%[107, 108]. Importantly, reproducible, moderate-severe biventricular systolic dysfunction was induced within six weeks of completion of DOX dosing. LV and RV EF declined from 46.2 $\pm$ 4.7% (baseline) to 31.3 $\pm$ 8.5% (final) ($p<0.01$) and from 39.5 $\pm$ 5.6% to 28.9 $\pm$ 9.6%, ($p<0.05$), respectively. Histological changes comprised cardiomyocyte degradation, vacuolisation, Purkinje fibre damage, small vessel injury and significantly, interstitial and replacement fibrosis of both ventricles and which were typical of pathological features associated with non-ischaemic cardiomyopathy[109-111].

1.7 Effects of Omega-3 PUFA in an Ovine Model of DOX-induced Cardiomyopathy

Based on the validation study findings, sheep may represent a suitable large animal model for the assessment of the cardioprotective effects of omega-3 PUFA supplementation in response to Doxorubicin administration. Ruminants have been shown to consume omega-3 PUFA supplemented feed (including fish products) and absorb these into skeletal muscle and other tissues[112-114]. These studies were undertaken to investigate increasing the omega-3 PUFA content of skeletal muscle as a dietary source for human consumption. Some reduction in absorption compared with humans due to hydrogenation by ruminal bacteria has been reported[115], but this may be countered by administering higher doses. Modest increases in tissue omega-3 PUFA have been demonstrated in sheep in response to dietary supplementation with fish oil, and with factors such as dose and breed of sheep affecting the level of absorption of omega-3 PUFA into tissues[113, 114]. Merino sheep have not been previously assessed for their uptake of dietary fish oil, nor have any ovine studies characterised the biodistribution of long chain fatty acids in the myocardium after supplemental therapy.

1.7.1 Thesis Studies Proposal

This thesis therefore consisted of two sub-studies:

Study 1 was designed to verify the feasibility of increasing plasma and myocardial phopholipid membrane bound uptake of EPA and DHA in Merino sheep following oral ingestion of fish oil. In turn, this enabled us to investigate the cardiac effects of

dietary omega-3 PUFA on in sheep also receiving intracoronary doxorubicin dosing (**Study 2)**.

1.7.1.1 Study Hypotheses

- Study 1: *"Oral ingestion of fish oil results in a significant increase of omega-3 PUFA levels into plasma, and erythrocyte and cardiomyocyte cell membranes"*
- Study 2, *"Dietary supplementation of omega-3 PUFAs will protect myocardium from DOX-induced cardiomyopathy, and this will manifest as less reduction in Left Ventricular Ejection Fraction compared with control group"*.

Materials and Methods

Animal ethics approval and study management

The full study proposal was submitted to the Institute of Medical & Veterinary Science and The University of Adelaide Animal Ethics Committees (AEC). Approval was granted by the IMVS (Approval No. 105/07, 13th August) and University AEC's (No. M-043-2007, 5th September 2007) for the use of 26 animals to conduct the proposed studies. The use of additional scavenge myocardial tissue from four healthy weight-matched animals was also approved to provide myocardium for comparative histology.

Use of Animals and Study Management

The studies were undertaken over a 20 month period from October 2007 to June 2009. Individual sheep were identified by a colour coded and numbered ear tag. The first 10 animals were used in a pilot study to further refine the DOX infusion protocol. The pilot study assessed the feasibility of further reducing the number of DOX dosings (compared with the earlier validaton study), and accordingly, the number of animal interventions, whilst maintaining induction of moderate levels of cardiac dysfunction with acceptable mortality rate. Five of these animals firstly received Omega-3 PUFA drenching to verify uptake of omega-3 PUFA into plasma and tissues. The remaining five sheep were fed normal diet.

Once the infusion protocol was established, a further 16 animals, in two batches of eight, were used for the subsequent main study. These were randomly allocated to fish oil (omega-3 group) or olive oil placebo drenching (control group) for the study duration. Sheep with poor baseline ventricular function as assessed by

echocardiography and CMR were not included into the DOX infusion protocol. DOX-infusion data is reported from the remaining 17 animals (eight omega-3 and nine control group sheep) and includes data from some animals in the pilot as well as the main study.

2.1 Omega-3 PUFA Dosing Study

2.1.1 Drenching Protocol

Five skeletally mature Merino wether sheep (weight range 71 to 76kg) were drenched three times per week for up to 20 weeks (50mL Mini Drenching Gun) with 23mLs of fish oil containing 300mg/mL EPA + DHA. This equates to approximately 10g of fish oil and comprises 3g of long chain omega-3 PUFA (EPA+DHA) intake per day. For the main study, weight and age matched control sheep were drenched with an identical volume and frequency with olive oil placebo. Lucerne chaff and water was supplied *ad lib* to both groups.

2.1.2 Collection of samples for fatty acid level assessment

All fatty acid analyses were conducted by the Food and Nutrition Group, School of Agriculture, Food and Wine, University of Adelaide. Blood samples were taken from each sheep prior to commencement of drenching, then weekly for four weeks and then at monthly intervals thereafter and finally, at time of euthanasia, to monitor changes in erythrocyte membrane-bound levels of omega-3 PUFA. Following euthanasia, the sheep were exsanguinated and the heart removed intact. Approximately 3mm^3 samples of myocardium were taken from each of the left and right ventricles adjacent to the apical tip, and left and right atrial appendage and stored at -20C for assessment of myocardial fatty acid levels[88].

2.1.2.1 Myocardial sample preparation

Myocardial samples were cleaned of adipose tissue and clotted blood. Approximately 0.2 g tissue was homogenized in 2mL saline before mixing with 3 mL methanol. Chloroform (6mL) was added, the samples were centrifuged (1560 x *g*, 10 min, room temperature), and the chloroform phase was transferred to a 20-mL glass vial and evaporated to dryness

2.1.2.2 Blood sample preparation

Whole blood was centrifuged at 1800g for 10 minutes, and the plasma removed and stored at -70^{o}C until analysis. The remaining red blood cells (RBC) were washed 3 times with normal saline, then 1 ml of RBCs was added to 0.5 ml saline and 2 ml propan-2-ol, vortex mixed and allowed to stand for 5 minutes, 4 ml chloroform was then added and mixed thoroughly. Following centrifugation, the lower chloroform layer containing the total lipid fraction was removed and stored in a glass vial at -20^{o}C until analysis. Total lipids were extracted from plasma with chloroform/ methanol (2:1, by volume).

2.1.2.3 Separation of phospholipids, preparation of fatty acid methyl esters and identification by gas chromatography

Phospholipids were separated from total lipid extracts from each of the myocardial, plasma and RBC preparations by thin-layer chromatography (TLC Silica gel 60H Merck Darmstadt, Germany). The TLC solvent system was petroleum spirit/diethyl ether/glacial acetic acid (180:30:2, by volume). Lipid classes were visualised with fluorescein 5-isothiocyanate against TLC standard 18-5 (NuChek Prep Inc: Elysian,

MN, USA). All solvents contained the anti-oxidant butylated hydroxyl anisole at 0.005% (wt/vol). Phospholipid fractions were transesterified by methanolysis (1% H2SO4 in methanol) for 3 h at 70° C. After cooling, the resulting fatty acid methyl esters (FAME) were extracted with n-heptane and transferred into gas chromatography vials containing anhydrous Na_2SO4. FAME were separated and quantified with a Hewlett-Packard 5880 gasliquid chromatograph using a capillary column equipped with flame ionisation detection and Hewlett-Packard Chem-Station data system (Avondale PA, USA). Separation was achieved on a 50 m x 0.33 mm ID BPX-70 column (SGE, Melbourne, Australia). Helium was the carrier gas at a column flow rate of 35 cm/s. The inlet split ratio was set at 30:1. The oven temperature at injection was set at 140℃ and programmed to rise to 220℃ at 5C/min. The injector and detector temperatures were set at 250℃ and 300℃, respectively. FAME were identified by comparison of retention times to authentic lipid standards (NuChek Prep Inc: Elysian, MN. USA)[116].

2.2 Ovine Model of DOX-induced Cardiomyopathy

2.2.1 General Anaesthesia

Animals were fasted for 24 hours prior to general anaesthesia, which was carried out for pericardial window, CMR, TTE, DOX infusion protocols and prior to euthanasia. Anaesthetic induction was achieved via masking with a mixture of isoflurane (5%) in 100% oxygen by endotracheal intubation. Anaesthesia was maintained by inhalation of a mixture of isoflurane (2-3%) in 100% oxygen. Animals were mechanically ventilated with a tidal volume of 10mL/kg to maintain a partial pressure of expired carbon dioxide of approximately 40mmHg. Additional

monitoring under anaesthesia included pulse oximetry, continuous electrocardiography, and intra-arterial assessment of blood pressure.

2.2.2 Pericardial Windows

Sheep received a pericardial window procedure prior to intra-coronary DOX-infusion protocol to avoid inflammatory pericardial effusion in response to DOX[117]. Following anaesthetic induction, sheep were placed in left lateral recumbency and a $20cm^2$ area over the left rib cage was shaved and scrubbed (Betadine sponge and 70% alcohol solution). A 15cm length incision was made over the 6^{th} intercostal space and dissection proceeded through the underlying fascia and muscle layers. Manual ventilation was taken over during initial dissection. Haemostasis of subcutaneous tissues continued as the intercostal membrane was perforated. A 3cm diameter segment of the pericardium was then resected. Four x 2.0 Polysorb sutures were used to re-appose the intercostal margins. Just prior to final closure of the thoracic cavity by tensioning the final Polysorb suture, the lungs were maximally inflated to reinstate normal baro-physiology. Subcutaneous and epidermal layers were then closed using 0.0 Polysorb. Animals were recovered quickly without insertion of a chest tube. One millilitre ketoprofen (Ilium Co. NSW, Australia) and 1mL/10kg Teramycin (Pfizer Co. Pty Ltd, Sao Paulo, Brasil) was administered IM for analgesia and antibiotic prophylaxis respectively. IM Ketoprofen was continued once daily for 2-3 days.

2.2.3 Cardiac Magnetic Resonance Imaging

The primary assessment of cardiac contractile function was via three lead electrogram ECG-gated CMR to assess global and regional left ventricular ejection

fraction (LVEF). Sheep underwent CMR two weeks following pericardial window and then at 12 weeks following final DOX infusion to establish baseline and final LVEF and end-diastolic and end-systolic volumes (EDV, ESV). Prior to CMR, sheep were anaesthetised and the wool over the left para-sternal region shaved and then wiped with 70% ethanol solution to improve adherence of the ECG leads. The sheep were then transferred to the MRI suite and positioned in supine recumbency inside the scanner (Siemens Sonata 1.5 Tesla MR Imaging system, Siemens, Erlangen, Germany). Cine images were ECG-gated and consisted of steady state free precession (SSFP) sequences with the following parameters: repetition time (T/R)/echo time (TE) 52.05 ms/1.74ms; flip angle 70^{o}; matrix 256 x 150; 25 phases per cardiac cycle; FOV 380mm with slice thickness of 6mm with a 4mm inter-slice gap.

2.2.3.1 Measurement of Left Ventricular Ejection Fraction

Ventricular ejection fractions and chamber volumes were determined in blinded fashion, with Argus software (Siemens Medical Solutions, Erlangen, Germany). End-diastolic and end-systolic images were chosen as the maximal and minimal, mid-ventricular, cross-sectional areas in a cinematic display. Short axis endocardial and epicardial borders were traced manually for each slice in end-diastole and end-systole. As per modified Simpson's rule, these areas were multiplied by the slice thickness (10 mm) and added together to obtain the EDV and ESV, respectively. Papillary muscles were included for the volume measurements. Atrial slices at end-systole secondary to apical movement of the base of the heart during LV contraction were not included. Ejection fraction was calculated by the formula:

$$EF\ (\%) = (EDV\text{-}ESV) \ / \ (EDV \times 100)$$

2.2.4 Transthoracic Echocardiogram

Transthoracic echocardiogram (TTE) was performed prior to each DOX infusion and then at time of euthanasia, to enable serial monitoring of changes to contractile function during the study period. Echocardiography was performed 30 minutes after anaesthetic induction and the commencement of mechanical ventilation. Sheep were positioned in right lateral recumbency and two dimensional-guided M-mode measurements of the LV end-diastolic diameter (EDD) and end-systolic diameter (ESD) were taken from right parasternal short axis views, just basal to the insertion of papillary muscles. Data was averaged from three separate consecutive cardiac cycles. Left ventricular fractional shortening (FS) was derived from the equation;

$$FS\ (\%) = \frac{(EDD - ESD)}{EDD \times 100}$$

2.2.5 Blood Samples

Peripheral venous blood samples were taken immediately before and then 24 hours following each DOX infusion, and then just prior to euthanasia to quantify full blood count, renal and liver function and cardiac biomarkers (troponin-T and creatinine kinase).

2.2.6 DOX-Infusion Protocol

2.2.6.1 Establishment of dosage

The DOX dosing strategy was established during the pilot study. Administration of 0.75mg/kg per dose was used in accordance with previously published data[117]. Administration of three and up to four fortnightly doses of 0.75mg/kg DOX did not achieve the intended degree of cardiac dysfunction in the pilot study control animals as based on echocardiography and CMR findings. Further validation revealed that

the optimal cumulative dose to result in moderate cardiac dysfunction and acceptable animal mortality and morbidity comprised three doses of 1.2mg/kg/dose at fortnightly intervals, (cumulative dose of 3.6mg/kg), followed by a 12 week period prior to euthanasia. This was the adopted DOX regimen for the study. In total, 17 Merino wether sheep (mean weight 55.6 $\pm$ 7.1kg) received this DOX-infusion protocol.

2.2.6.2 Group allocation

For the main study, sheep were randomly allocated to receive either 23mls olive oil (placebo) or fish oil (treatment group), 23mLs three times per week via drenching throughout the DOX dosing and follow up period, until time of euthanasia. Both groups were matched for body size and weight. Results from the pilot drenching study demonstrated that erythrocyte membrane bound concentrations of long chain fatty acids increased by 2-3 fold within two to three weeks of dietary fish oil supplementation. Therefore, omega-3 PUFA drenching was commenced two to three weeks before the start of the DOX protocol, and continued throughout the dosing and follow-up period. A higher concentration (60% - 600mg/mL EPA+DHA) of omega-3 PUFA was used during the first two weeks drenching period to expedite elevation of phospholipd membrane bound EPA+DHA prior to commencement of DOX infusions. A 30% concentration (300mg/mL EPA+DHA was then used for the remainder of the study period.

2.2.6.3 Catheterisation and DOX-infusion

Following anaesthetic induction, sheep were placed in supine recumbency and the groin region shaved, sterile scrubbed and draped. Peripheral arterial access was

obtained via percutaneous cannulation of the right femoral artery using 5-French introducer and sheath (Cordis Corp, Miami, USA). Heparin (100 IU/kg) Heparin was administered via 5mL bolus into the arterial sheath. An Amplatz diagnostic catheter (AL1 Cordis Corp, Miami, USA) was used to catheterise the left-sided coronary arteries under fluoroscopic guidance (Phillips BV25 Fluoroscopic Imager, Bloomfield CT, USA). Coronary artery engagement was confirmed by bolus administration of iodinated contrast media (Ultra-vist 360 Bayer Ltd, NSW Australia). The left anterior descending and circumflex arteries were engaged selectively, with half of the DOX administered to each artery in sequence. The total dose of DOX (1.2mg/kg made up in 50mL 0.9% saline) was infused over a 30 minute period. During dosing, the catheter position was repeatedly visualised by fluoroscopy and records taken of heart rate, intra-arterial blood pressure, and electrogram appearance.

When dosing had been completed and the catheter removed, femoral artery haemostasis was achieved by manual compression for at least 10 minutes. Animals received post-operative antibiotic prophylaxis and analgaesia for 72 hours. Sheep were monitored in a small (1m x 2m) housing pen for the duration of the DOX dosing protocol. Two weeks after the final DOX infusion they were returned to the field station for a further 10 weeks until final CMR and euthanasia. Animals were monitored twice daily for health status and condition.

2.2.7 Retrieval

At 12 weeks following final DOX infusion, and within 3-4 days of final CMR, sheep were anaesthetised and final blood samples, TTE and haemodynamic measurements obtained. Euthanasia was then performed by intravenous

administration of 120mg/kg pentobarbital. The heart was removed intact and the pericardial fat layer removed. Surface areas of pallor or other notable features were documented. Approximately $3mm^3$ samples of ventricular and atrial appendage myocardium were taken and stored at -20C for subsequent assessment of omega-3 PUFA levels. The heart was then weighed intact and immediately perfusion fixed with 3-4L of 4% paraformaldehyde, before storage in 10% buffered formalin. For histological comparison, hearts were also obtained and processed identically from four healthy control sheep.

2.2.8 Histopathology Protocol

Transverse slices of the fixed heart were made at 10mm intervals starting at the LV apex. Slices from the basal, mid and distal ventricular levels were divided into six, six and four segments respectively. Segments were labelled according to their anatomical orientation; anterior, posterior, lateral and septal aspects, and then processed for paraffin embedding. The apical segment was processed whole. Sections (10*u*m) were taken from each paraffin block and stained with haematoxylin and eosin for assessment of morphologic cellular changes and van Gieson stain for identification and quantification of fibrotic tissue.

2.2.9 Histological Assessment of Percent Area Fibrosis

The van Gieson stained slide sections were photographed with a NanoZoomer Digital Pathology system (Hamamatsu Photonics, Hamamtsu, Japan). Complete image sets were exported from each slide section at 5x magnification into Image Pro-Plus software (ver 5.2.1.59, Media Cybernetics Inc. Bethesda, MD USA). The percentage area of fibrosis occupying each segment was calculated using “colour

cube-based segmentation" algorithm to select and quantify fibrotic tissue (staining red) against normal myocardium (brown). The epicardium and surface coronary vessels contain abundant collagen in normal healthy myocardium, and were therefore removed from the analysis region of interest. Data are presented as segmental and mean total % fibrosis area for each of the Omega-3, placebo control, and healthy group.

2.2.10 Sample size calculation

A study group totaling 16 animals achieves a statistical power of 80% to detect a 12% absolute difference in decline in mean global left ventricular ejection fraction on CMR between sheep in each group, (with 8% variance (SD)) and a p value < 0.05. Allowance was made for two animals per group not surviving the disease process.

2.2.11 Statistical Analysis

For the Omega-3 PUFA dosing study, statistical analysis was performed using Prism Graph ver. 5.03 (Graph Pad Software, La Jolla, Ca, USA). Normality of data was assessed by Shapiro-Wilk test and normally distributed data presented as mean $\pm$ S.D. Intergroup differences at baseline were assessed using Students unpaired t-test. Comparisons between groups over time were performed by two way ANOVA. Statistical significance was established at a two-tailed p value <0.05. Where a significant difference was encountered, this was post-tested using Tukey-Kramer Multiple comparisons test.

For the DOX-infusion study, statistical analysis was performed using SPSS ver 15 (IBM Corporation, Route 100, Somers NY USA). Normally distributed variables

are reported as mean ± SD. Intergroup baseline comparisons were analysed by using Student's unpaired, two-tailed t-test. Skewed datasets are reported as median and inter-quartile range. Categorical variables are reported as number and percentage. Statistical significance was set at two-tailed $p<0.05$.

Continuous variables were assessed using linear mixed effects models to examine the effects of time (pre, post or baseline, dox1, dox2, dox3), group (placebo, Omega-3) and their interaction (group*time). A random effect of sheep ID was fitted to the model to account for the repeated observations (time). The residuals were visually assessed for normality to ensure appropriate model fit. If the residuals were skewed, the data was log transformed and the model re-run. If the interaction term was significant, appropriate post-hoc tests were performed within groups. If mixed effects model Hessian matrix was not satisfied because of limited follow-up data (due to unexpected deaths or technical issues) statistical analysis was not performed, but raw data presented. Simple inter or intra-group comparisons were performed using t-tests for normally distributed data, Mann-Whitney for skewed data or Chi-squared for count data.

Intraclass agreement for the LVEF and % area of fibrosis measurements were assessed via intraclass correlation and 95% Bland Altman confidence limits of agreement (Appendix).

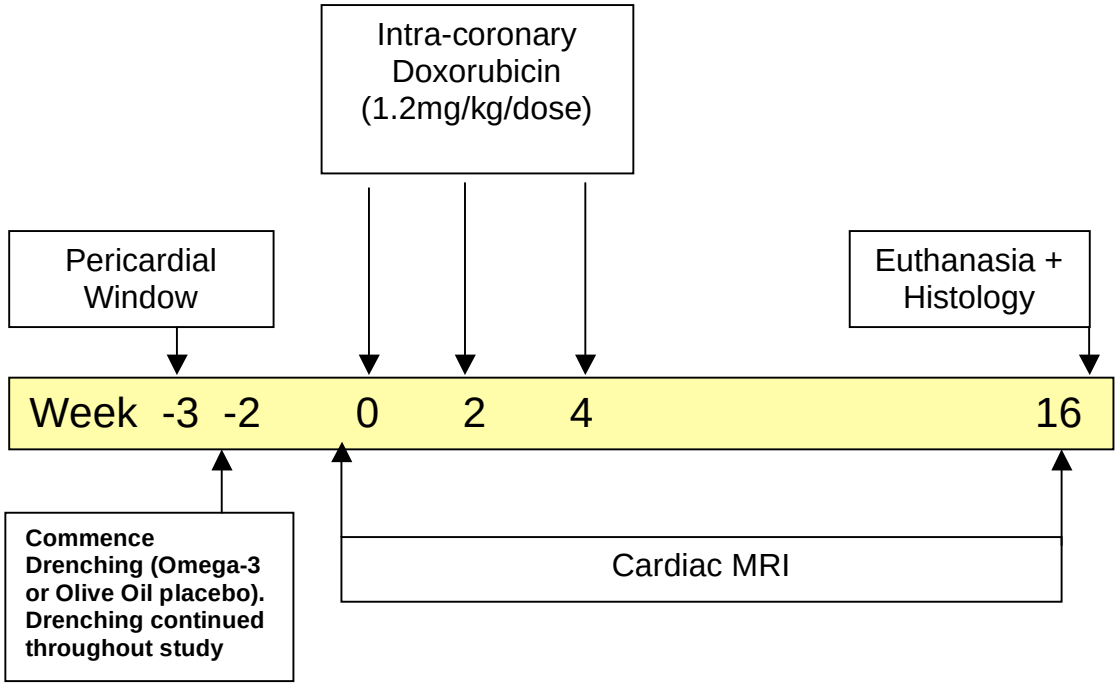


Figure 2.1 Flow Chart of Study Protocol

Table 2.2 Equipment and Supplier

Equipment	Specifications and Supplier
Drenching	
Drenching Gun	Mini 50mL Drenching Gun – Farmers Mailbox, Victoria, Australia
Omega-3 Fatty Acid (Fish Oil) 60% preparation	Berg Lipidtech, Eidsnes, Norway
Omega-3 PUFA (Fish Oil) 30% preparation	Melrose Laboratories Pty Ltd. Mitcham, Victoria, Australia
Olive Oil (Placebo)	Melrose Laboratories Pty Ltd. Mitcham, Victoria, Australia
Fatty Acid Level Analysis	
Silica gel 60H	Merck, Darmstadt Germany
TLC standard 18-5	NuChek Prep Inc: Elysian, MN, USA
BPX-70 column	SGE, Melbourne, Australia
Lipid standards	NuChek Prep Inc: Elysian, MN, USA
Gasliquid Chromatograph and Chem-Station data system	Avondale PA, USA
Imaging and Analysis	
Magnetic Resonance Imaging Scanner	Siemens Sonata 1.5 Tesla MR Imaging system, Siemens, Erlangen, Germany
Echocardiography Machine	Siemens Acuson console, Erlangen, Germany
Fluoroscopic Imager	Phillips BV 25, Bloomfield CT, USA
Argus software	Leonardo workstation, Siemens Medica Solutions, Erlangen, Germany
NanoZoomer Digital Pathology system	Hamamatsu Photonics, Hamamatsu Japan

Equipment	Specifications and Supplier
Image Pro Plus ver 5.2.1.59	Media Cybernetics Inc. Bethesda, MD USA
Anaesthesia and Euthanasia	
Isoflurane	Veterinary Companies of Australia Pty Ltd, Kings Parkm NY USA
Pericardial Windows, Peri-operative medications	
Doxorubicin Hydrochloride	Pfizer Inc, New York, NY, USA. Made up in 0.9% saline by Royal Adelaide Hospital Pharmacy.
Ketoprofen	Ilium Brand, Troy Laboratories, Smithfield NSW, Australia
Terramycin	Pfizer Inc, New York, NY, USA
Furosemide	Ilium Brand, Troy Laboratories, Smithfield NSW, Australia
Catheterisation	
Heparin	5,000 IU in 5mL saline. Pfizer Pty Ltd, West Ryde, NSW. Australia.
5 French introducer sheath	Cordis Corporation, Johnson and Johnson, Miami, FL, USA
5 and 7 French AL 1 and 2 Catheters	Cordis Corporation, Johnson and Johnson, Miami, FL, USA
Contrast Solution	Ultravist-370, Bayer Australia, Pymble, NSW, Australia.

Results

3.1 Omega-3 PUFA Dosing Study

Omega PUFA level data is presented as a percentage of total fatty acid content within the plasma, erythrocyte (RBC) or myocardial membrane bound fatty acid as indicated.

3.1.1 Omega-3 PUFA Levels

3.1.1.1 Omega-3 PUFA Baseline levels

At baseline, the plasma levels of total omega-3 long chain PUFA were significantly higher in the dosing study sheep assigned to the control group than in the omega-3 group, 14.89 ± 1.70 % Control Group Dosing study (n=4) vs 7.26 ± 0.94% Omega-3 dosing study sheep (n=4) ($p<0.05$ ANOVA, with post test Tukey Multiple Comparison Test)), Table 3.1.1.

The baseline erythrocyte (RBC) membrane bound levels of total omega-3 long chain PUFA were also significantly higher in the dosing study sheep assigned to the control group, compared with those in the dosing study omega-3 group; 2.08 ± 0.41% Control Group Dosing study (n=4) vs 1.57 ± 0.19% omega-3 group dosing study (n=4) ($p<0.05$ ANOVA, with post test Tukey Multiple Comparison Test)), Table 3.1.1.

Omega-3 Long Chain fatty acid (n-3 LC PUFA)	Omega-3 Group **Dosing Study (n=4)**	Control Group **Dosing Study (n=4)**
Baseline Plasma Phospholipids Levels		
18:3n-3 (ALA)[1]	5.95 ± 0.94[a]	2.32 ± 0.20[b]
20:5n-3 (EPA)[1]	1.71 ± 0.22[a]	2.76 ± 0.56[bc]
22:5n-3 (DPA)[2]	2.76 ± 0.21[a]	5.52 ± 1.00[c]
22:6n-3 (DHA)	2.79 ± 0.59[a]	6.61 ± 0.96[c]
Total n-3[2]	13.78 ± 0.73[a]	17.64 ± 1.53[b]
Total n-3 LC PUFA[1]	**7.26 ± 0.94[a]**	**14.89 ± 1.70[b]**
Baseline RBC Levels		
18:3n-3 (ALA)[2]	0.97 ± 0.14[ab]	1.15 ± 0.21[a]
20:5n-3 (EPA)[2]	0.65 ± 0.08[a]	0.63 ± 0.19[ab]
22:5n-3 (DPA)[2]	0.56 ± 0.07[a]	0.95 ± 0.21[b]
22:6n-3 (DHA) [2]	0.36 ± 0.09[a]	0.50 ± 0.08[a]
Total n-3[2]	2.78 ± 0.31[a]	3.52 ± 0.55[b]
Total n-3 LC PUFA[2]	**1.57 ± 0.19[ab]**	**2.08 ± 0.41[c]**

Table 3.1.1 Plasma and erythrocyte bound (RBC) baseline levels of long chain omega-3 PUFA. Plasma and RBC total long chain omega-3 PUFA were higher at baseline in the Dosing study control compared with the omega-3 group sheep ($p<0.05$). Values within a row with a different superscript are significantly different ($p<0.05$, ANOVA with post test with Tukey-Kramer Multiple Comparisons Test).

3.1.1.2 Omega-3 PUFA drenching study – erythrocyte membrane bound levels

Drenching sheep with 23mLs fish oil containing 300mg/mL EPA+DHA three times per week caused a significant increase in erythrocyte membrane bound levels of omega-3 long chain PUFA compared with olive oil fed placebo controls, ($p<0.05$ ANOVA, with post test Tukey Multiple Comparison Test), **(Figure 3.1.2)**. The increase in omega-3 PUFA levels reached significance between three to four weeks of drenching with fish oil and then stabilized following an additional three to four weeks of drenching. The plasma levels of omega-3 PUFA did not change in the control sheep (receiving olive oil placebo) over the duration of the study ($p>0.05$).

3.1.1.3 Omega-3 PUFA drenching study - myocardial levels

Samples of left and right ventricular (LV/RV) and right atrial tissue (RA) were harvested from the sheep hearts at time of sacrifice for assessment of myocardial phospholipid bound levels of omega-3 PUFA. There was a significant, approximately two-fold increase in the myocardial levels of omega-3 PUFA in myocardium taken from the omega-3 group sheep compared with controls ($p <0.05$), **(Figure 3.1.3)**.

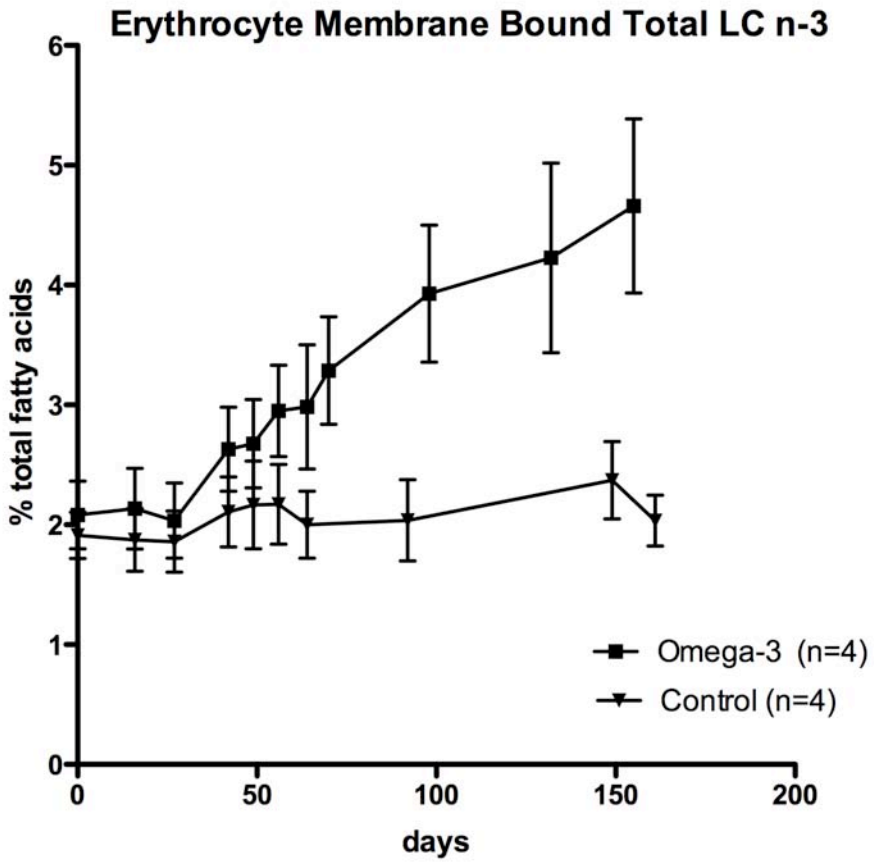


Figure 3.1.2 Changes in total erythrocyte membrane bound levels of omega-3 long chain PUFA levels over the study duration. The first three data points for each group were from blood samples taken prior to drenching, which commenced immediately following. By three to four weeks of drenching, there were significantly higher levels of erythrocyte membrane bound omega-3 long chain PUFA in the omega-3 group sheep compared with olive oil fed controls, and which continued until completion of the study, ($p<0.05$ ANOVA, with post test Tukey Multiple Comparison Test).

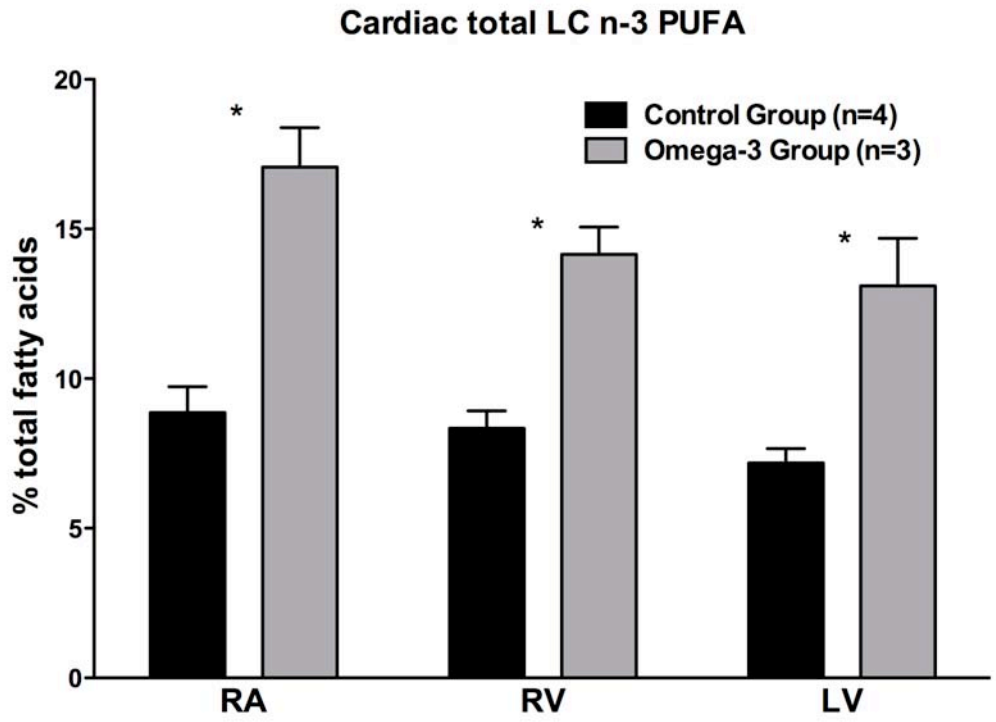


Figure 3.1.3 Total long chain myocardial phospholipid bound omega-3 PUFA levels in tissue samples taken from the right atria (RA) and left and right ventricle (RV/LV) at time of euthanasia. Myocardial omega-3 PUFA levels were approximately two-fold higher in the omega-3 group sheep than in the olive oil fed controls. This applied to all cardiac regions analysed, * ($p < 0.05$).

3.1.1.4 Myocardial arachidonic acid levels

Myocardial phospholipid bound levels of omega-6 arachidonic acid in the left and right ventricular (LV/RV) and right atrial (RA) tissue harvested from the sheep hearts at time of sacrifice were assessed. There was a significant, approximately 30% reduction in the myocardial levels of arachidonic acid in myocardium from each of the three sites sampled in the omega-3 group sheep compared with controls ($p<0.05$), **(Figure 3.1.4)**.

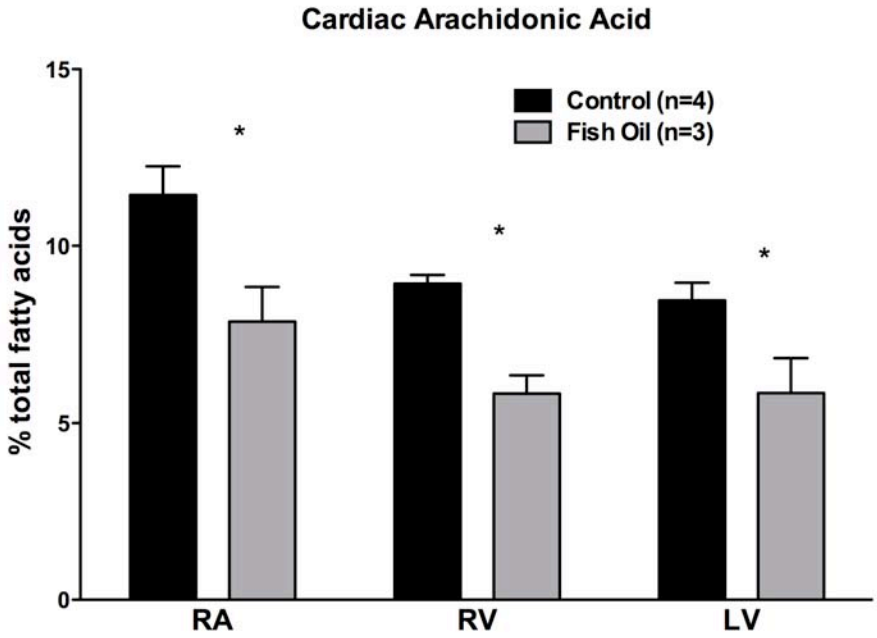


Figure 3.1.4 Myocardial bound omega-6 arachidonic acid levels from right atria and left and right ventricular samples taken from sheep myocardium at time of euthanasia. There was a significant, approximately 30% reduction in arachidonic acid levels in myocardium from omega-3 group sheep compared with controls at each of the three sites sampled ($^{*}p<0.05$).

3.2 Ovine DOX-infusion Study

3.2.1 Clinical Results

3.2.1.1 Mortality rate

A total of 17 animals (weight range 41 to 62.5kg) received the intra-coronary DOX infusion protocol (eight omega-3 and nine placebo fed controls). The baseline parameters for these animals are shown in Table 3.2.1. There were three premature deaths in each group, (omega-3 treated (37.5%) and control groups (33%)). The control group deaths occurred between three and 12 weeks following the final scheduled DOX dosing. One animal died suddenly in its holding pen 12 weeks following the final DOX infusion and hours prior to the final scheduled CMR. This

animal had appeared well in the weeks following DOX dosing. The sudden nature of its death implicated an arrhythmic cause. The second sheep that died early appeared in good condition during the weeks following its final DOX dose, but died suddenly in its field pen at six weeks post DOX 3. The absence of preceding signs and the sudden nature of its death also suggested ventricular arrhythmia (VA) as cause of death. The heart and lungs were retrieved for histopathology assessment.

The third placebo sheep developed malaise at three weeks following its final DOX dose. The animal appeared in discomfort and ceased feeding. A provisional diagnosis of fluid retention secondary to cardiac failure was made. Fluid was withheld overnight and 3 doses of furosemide (50mg) were administered throughout the following day to diurese the animal of excess fluid. The sheep's condition showed initial improvement, but 24 hours later the animal suffered a witnessed cardiac arrest and could not be revived. The heart was removed intact for histological assessment.

In the omega-3 group, two animals died suddenly, one at six and the other at seven weeks following final DOX dose. In both cases, there were no preceding signs of cardiac failure nor non-cardiac pathology, indicating sudden cardiac arrhythmia as the likely cause of death. The third premature death in the omega-3 cohort occurred as a result of procedural complications during the final DOX dosing.

3.2.1.2 Electrocardiographic changes

Electrocardiogram changes were frequently observed during intra-coronary DOX infusion in sheep in both groups. Most frequent changes consisted of transient ST segment elevation or depression. Infusions were never associated with ectopic beats

nor arrhythmia and ECG changes always resolved prior to completion of the angiographic procedure.

	Control Group	Omega-3 Group
n	9	8
Weight Range	41 to 62.5kg	41 to 62kg
Mean B/L Weight (kg) (p=NS)	58.0 ± 14	53.0 ± 12
Mean B/L LVEF (%) (p=NS)	42.5 ± 2.3	48.6 ± 2.6
Mortality Rate	3/9 (33%)	3/8 (37.5%)
Likely cause of early deaths	Arrhythmia : (n=2 sheep) Congestive Heart Failure: (n=1)	Arrhythmia: (n=2). Procedural: (n=1)
Mean F/U Period for Survivors from DOX 1 (days)	89 ± 7 days	90 ± days (6.03 SEM)

Table 3.2.1 Baseline and follow up parameters for sheep in DOX-infusion study. At baseline there was no difference in mean Weight, nor LVEF (p=NS) between groups. B/L = Baseline, F/U = Follow Up.

3.2.2 Left Ventricular Ejection Fraction and Volume Changes as Assessed by CMR

The LV EF was similar between omega-3 and control group animals at baseline (p=NS). DOX-infusion resulted in a significant decline in LV EF for both omega-3 and control groups ($p<0.05$), however, the Omega 3 group declined to a greater extent (19.4% absolute reduction; (from 48.6 ± 7.4% baseline to 29.2 ± 6.9% final)) compared to the placebo controls (8.1% absolute reduction; (from 42.5 ± 6.8% baseline to 34.4 ± 7.5)), ($p<0.05$ for intergroup comparison), **(Figure 3.2.2A)**.

The LV EF changes were accompanied by a significant increase in LV End-Diastolic volume (EDV) from baseline (72.9 $\pm$ 18.0mLs) to final (110.3 $\pm$ 38.5mLs) in the Omega-3 group ($p<0.05$), with no change in the control EDV over time (baseline 75.3 $\pm$ 13.0 to final 73.3 $\pm$ 11.0), (p=NS). There was a significant difference in final EDV between groups ($p<0.05$), however there was no group effect on EDV change over time by two-way ANOVA (p=NS), **(Figure 3.2.2B)**.

Left ventricular ESV showed a significant increase in the omega-3 group (38.1 $\pm$ 12.8mLs baseline to 79.1 $\pm$ 33.0mLs final) ($p<0.05$) with no change over time in the control group (43.7 $\pm$ 10.7mLs baseline to 49.4 $\pm$ 9.7mLs final), (p=NS). Although there was a significant difference in final ESV between groups ($p<0.05$), there was no group effect on ESV over time, using two-way ANOVA (p=NS), **(Figure 3.2.2C)**.

Figure 3.2.3 shows CMR generated long and short axis TrueFISP images of the heart in a control sheep pre- and post DOX infusion, showing wall thinning and dilatation changes typical of DOX-induced cardiomyopathy.

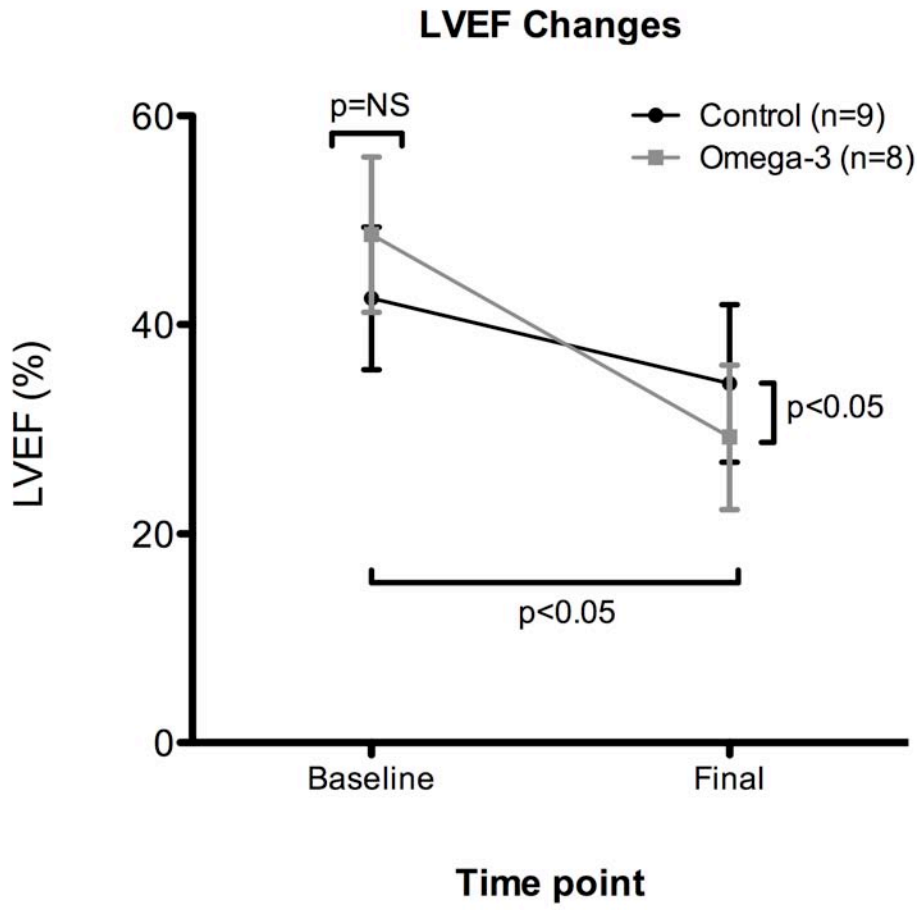
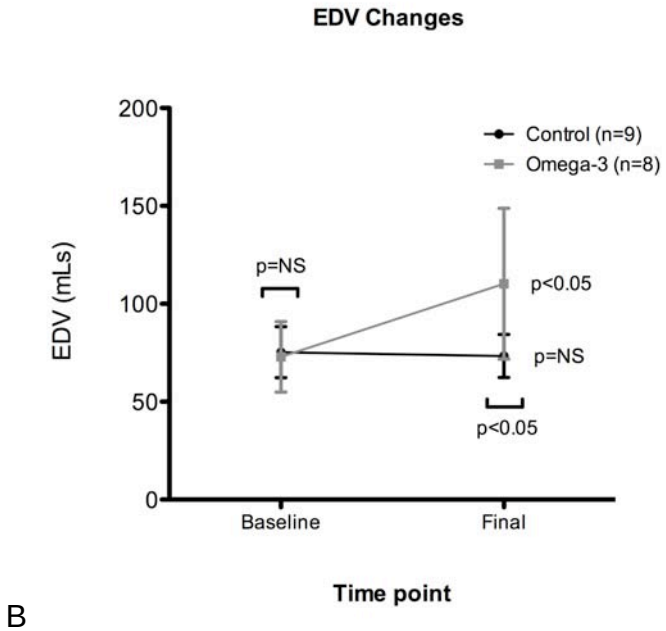
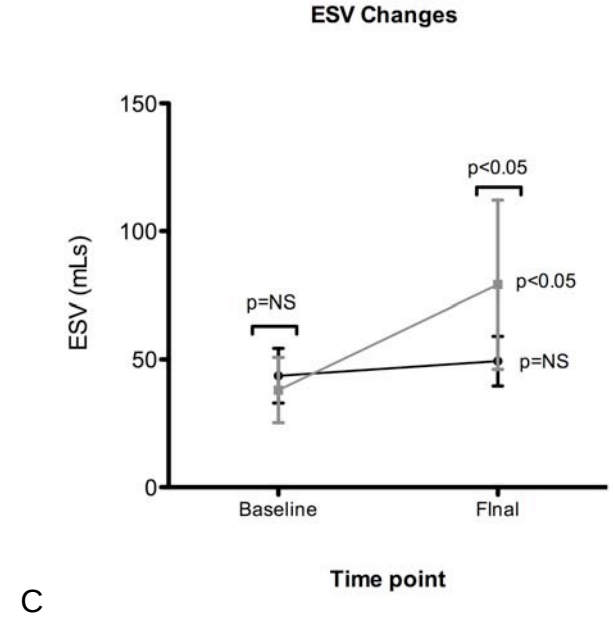


Figure 3.2.2A Changes in left ventricular ejection fraction (LV EF) in response to DOX-infusion protocol as assessed by CMR. LEVF was similar between groups at baseline (p=NS). There was a significant reduction in LV EF in both groups (p<0.05). Doxorubicin infusion resulted in a significantly greater mean absolute reduction in left ventricular ejection fraction (LV EF) in the omega-3 group compared with control group (p<0.05).

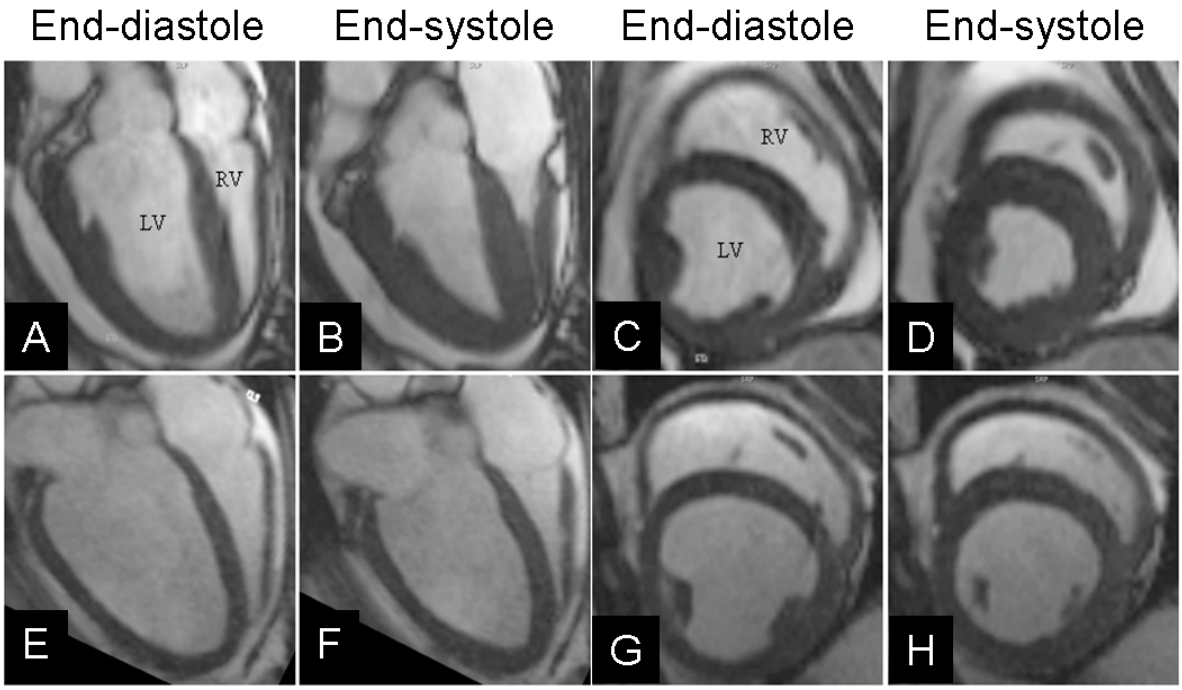


B



C

Figure 3.2.2 B and C. Changes in left ventricular end diastolic volume (EDV) (B) end systolic volume (ESV) (C) in response to DOX-infusion protocol. EDV increased significantly in the Omega-3 group, ($p<0.05$), with no change in the control EDV over time (p=NS). There was a significant difference in final EDV between groups ($p<0.05$). The LV End-Systolic volume (ESV) increased significantly in the omega-3 group with no change over time in the control group ($p<0.05$). There was a significant difference in final ESV between groups ($p<0.05$).



Supp. Fig. 4

Figure 3.2.3 Long axis (panels AB, EF) and short axis (CD, GH) TrueFISP CMR images of the heart at baseline (A-D) and final assessment (E-F) in a sheep with severe LV dilation and systolic dysfunction following DOX infusion protocol.

3.2.3 Fractional Shortening, End Diastole and End Systole Diameter Changes as assessed by Trans Thoracic Echocardiogram

Short axis subvalvular mean LV fractional shortening (SAX FS) was measured via echocardiogram prior to each DOX infusion and then immediately prior to sacrifice. Fractional shortening decreased significantly in response to DOX infusion in both groups (baseline FS 33.3 $\pm$ 4.5% to 20.1 $\pm$ 4.5% final in control group, and from baseline 34.4 $\pm$ 5.0% to 26.2 $\pm$ 7.7%, final in omega-3 group, ($p<0.01$). Significant deterioration of FS was not observed until after the final DOX infusion. There were no group nor time effect differences on FS when assessed by two-way ANOVA (p=NS). The changes in SAX FS following each DOX infusion are shown in **Figure 3.2.4.**

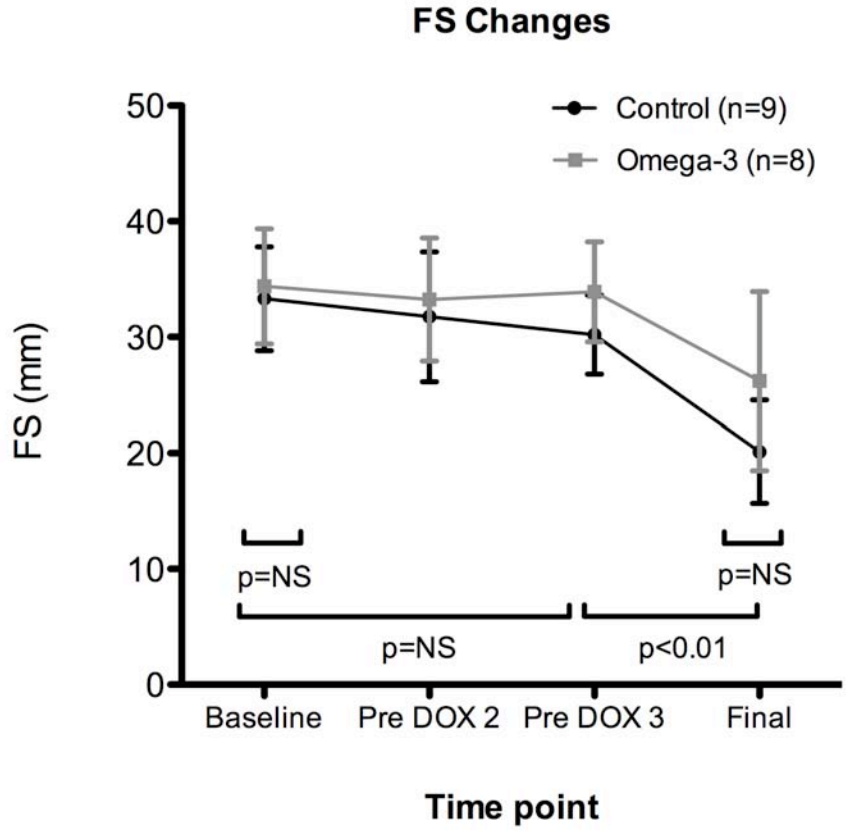


Figure 3.2.4. Left Ventricular Transthoracic echocardiography short axis Fractional Shortening Changes (FS). FS decreased significantly in response to DOX infusion in both the control and omega-3 groups, ($p<0.01$)). The decrease did not reach significance until following the third (final) DOX infusion. There were no group nor time effect differences in FS (p=NS).

3.2.4 Blood Results

Due to quarantine restrictions arising at the Large Animal Research Facility at Gilles Plains (LARIF) during the study, and where several of the study euthanasias were performed, final blood samples from one omega-3 group and five control group sheep could not be released for cell and serum marker levels analysis. Statistical analyses

was performed using endpoint data from the remaining animals, however the affect of the missing final data points in terms of statistical significance is unknown.

3.2.4.1 Troponin-T post DOX infusion

Elevations of serum troponin-T titer were generally mild following each DOX infusion, ranging from 0.03 to 0.66ug/L in the control group and 0.03 to 0.18ug/L in the omega-3 group animals. **(Figure 3.2.6A)**. Four (44%), five (56%) and four (44%) sheep in the Control group returned positive troponin-T levels following DOX 1, 2 and 3 infusions respectively. Six of eight sheep (75%) in the omega-3 group showed mild to moderate troponin-T rises following each of the three DOX infusions **(Figure 3.2.6B)**. Of these, five animals showed a troponin-T rise following each of the three DOX infusions.

3.2.4.2 Haemoglobin, white cell count and platelets

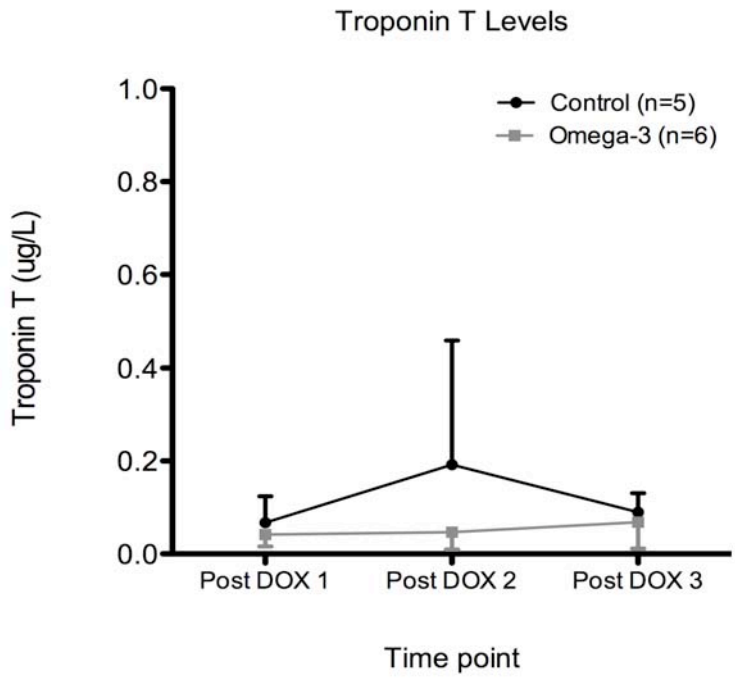
There were no group nor time effects on Haemoglobin levels in response to DOX infusion, p=NS, **(Figure 3.2.7A)**.

There were no group effects on White Cell Count (WCC), (p=NS). The WCC in both groups showed a non-significant decline following the first DOX infusion (p=NS), with a further significant decline following the second DOX infusion ($p<0.05$). The WCC levels then stabilised following the third and final DOX dose. The decline in WCC from baseline to final in both groups (time effect) was significant ($p<0.01$), **(Figure 3.2.7B)**.

The platelet count in both groups showed a significant decline following the first DOX infusion ($p=<0.05$), which then stabilised following the second and third DOX

infusion. The change in platelet count from baseline to final in both groups (time effect) was not significant (p=NS), **(Figure 3.2.7C)**.

A



B

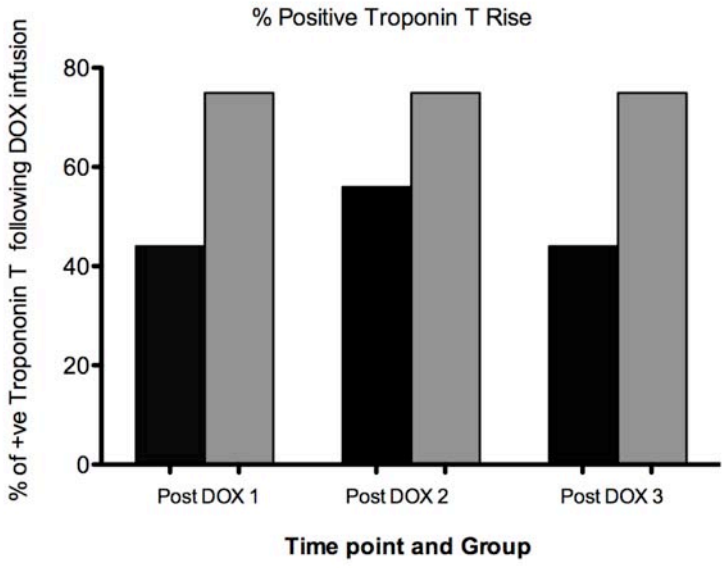


Figure 3.2.6 Mean Troponin-T levels post each DOX infusion for each group (A) and % positive Troponin-T rises following each DOX infusion for each group (B).

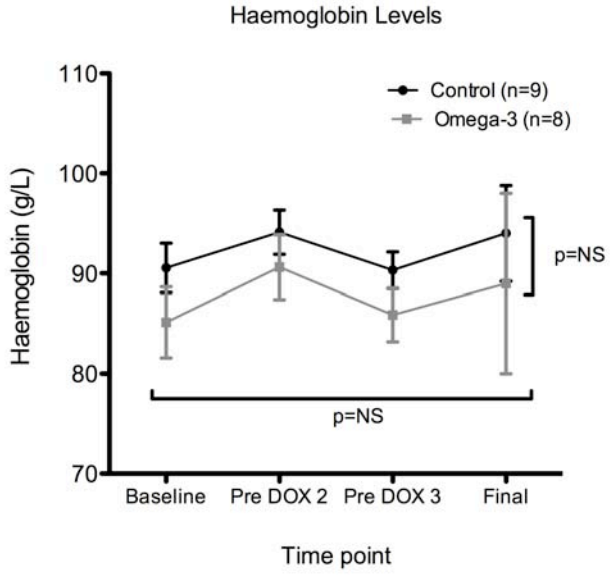


Figure 3.2.7A Changes in Haemoglobin levels (ug/L) following each DOX infusion. There were no group nor time effects on Haemoglobin levels in response to DOX infusion, (p=NS).

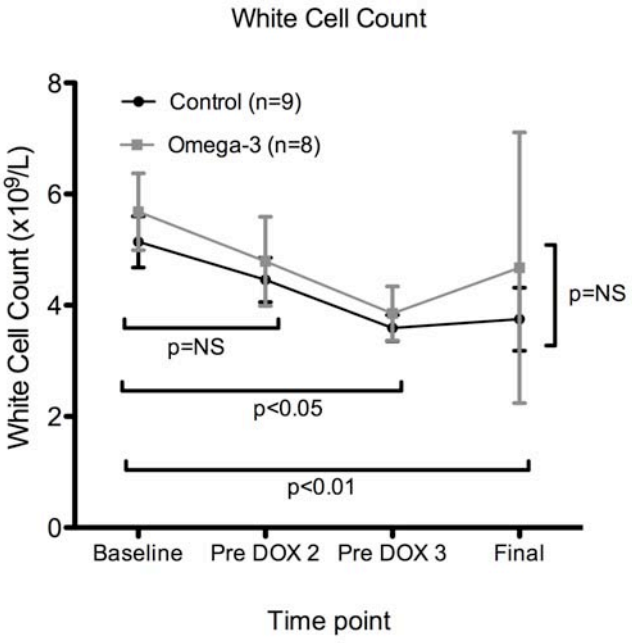


Figure 3.2.7B Changes in White Cell Count in response to DOX infusion. There were no group effects on White Cell Count (WCC), (p=NS). The WCC in both groups showed a non-significant decline following the first DOX infusion, and which declined further to significant levels following the second DOX infusion ($p<0.05$). The WCC levels then stabilised following the third and final DOX dose. The decline in WCC from baseline to final in both groups (time effect) was significant ($p<0.01$).

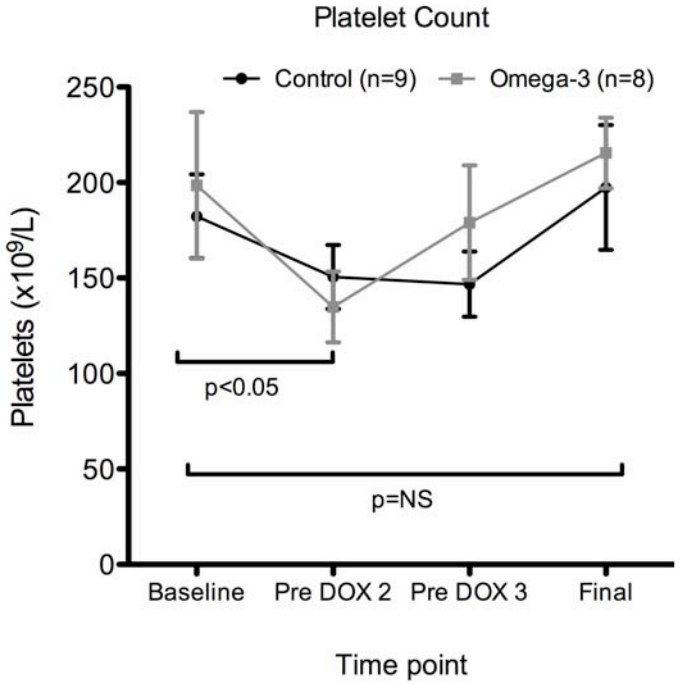


Figure 3.2.7C. Changes in platelet count in response to DOX infusion. The platelet count in both groups showed a significant decline following the first DOX infusion ($p<0.05$), which then stabilised following the second and third DOX infusion. The change in platelet count from baseline to final in both groups (time effect) was not significant.

3.2.5 Histopathological Assessment

3.2.5.1 Macroscopic appearances

At gross necropsy, in both groups, there were typically fibrinous adhesions to the pericardium and multiple areas of pallor were visible on both the epicardial and cut surfaces of the myocardium **(Figures 3.2.8A and B)**.

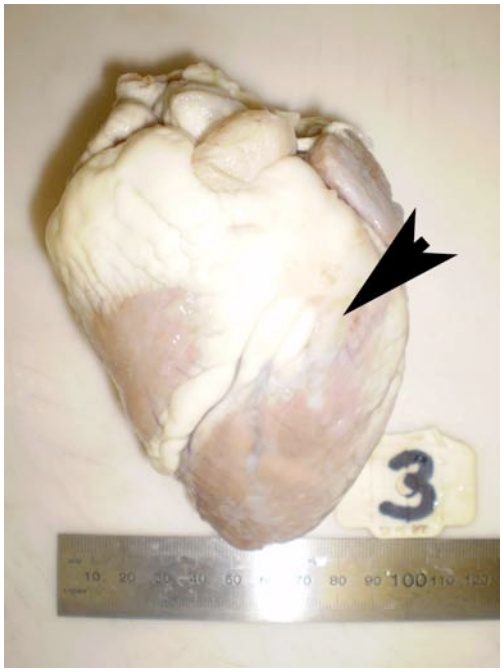


Figure 3.2.8A A formalin fixed intact ovine heart retrieved 12 weeks after three intra-coronary doses of 1.2mg/kg Doxorubicin. Large areas of surface pallor are clearly visible on the lateral surface of the left ventricle.

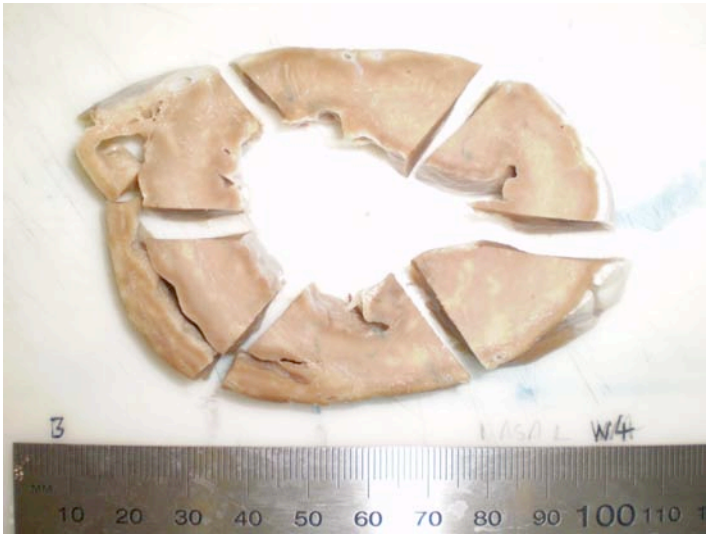


Figure 3.2.8B. Ventricular cut surface taken at basal (sub mitral valve) level, of Doxorubicin treated formalin fixed heart. This image shows the slicing protocol used for the mid and basal slices, ie: commencing clockwise from top segment; anterior, antero-lateral, postero-lateral, posterior, posterior-septal, anterior-septal. Foci of midmural pallor are clearly visible in each segment, particularly the anterior, antero-lateral and postero-lateral segments.

3.2.5.2 Histopathological findings

Histological review of Haematoxylin and Eosin stained slide sections from both groups revealed several pathological features typical of DOX-induced tissue damage. These included vacuolation within cardiomyocyte cytoplasm, loss of myofibrillar striations, areas of fibrosis with associated fibroblast proliferation and histiocyte infiltrate, vacuolization of purkinje fibre cells, occasional foci of necrotic cells and areas of perivascular oedema. Further changes included vasculitis, pyknosis of endothelial cells, suggestive of apoptosis, and mural lymphocyte infiltrates.

3.2.5.3 Ventricular fibrosis burden

Doxorubicin treatment resulted in the presence of both interstitial and replacement fibrosis distributed heterogeneously throughout the ventricular myocardium in both omega-3 and placebo animals. Foci of replacement fibrosis were typically small-moderate in size and were most frequently located in the sub-epicardial and/or mid-wall layers of the myocardium.

Quantification of the percent (%) area of fibrosis was determined from the van Gieson stained sections by using a colour cube based segmentation algorithm within Image Pro Plus software (ver 5.2.1) to select fibrotic tissue against normal myocardium, **(Figure 3.2.9)**.

The mean % area of fibrosis was calculated for each segment within each of the four myocardial levels sectioned from each heart ie: basal, mid, distal and apical levels. Mean % area of fibrosis was then calculated for each level, and also combined for all levels, and compared with normal healthy untreated myocardium. For the mean % area of fibrosis for all levels combined, there was a significantly greater % area of fibrosis in the placebo (3.73 $\pm$ 0.62%) and omega-3 (3.87 $\pm$ 1.91%) groups compared

with healthy normal myocardium (0.68 ± 0.68%), ($p>0.05$). However, there was no difference in % levels of fibrosis between the placebo and omega-3 group animals, ($p>0.99$), **(Figure 3.2.10A).** Furthermore, there was no difference in fibrosis levels in the control and omega-3 PUFA treated sheep at each of the four levels analysed; 3.63 ± 1.9% control basal, vs 3.65 ± 1.0% omega-3 basal, control mid 2.57 ± 1.6% vs 4.91 ± 3.1% omega-3 mid, control distal 3.32 ± 2.0 vs 2.70 ± 1.1% omega-3 distal, and control apical 4.0 ± 3.8 vs 4.8 ± 4.0 omega-3 apical, **(Figure 3.2.10B).**

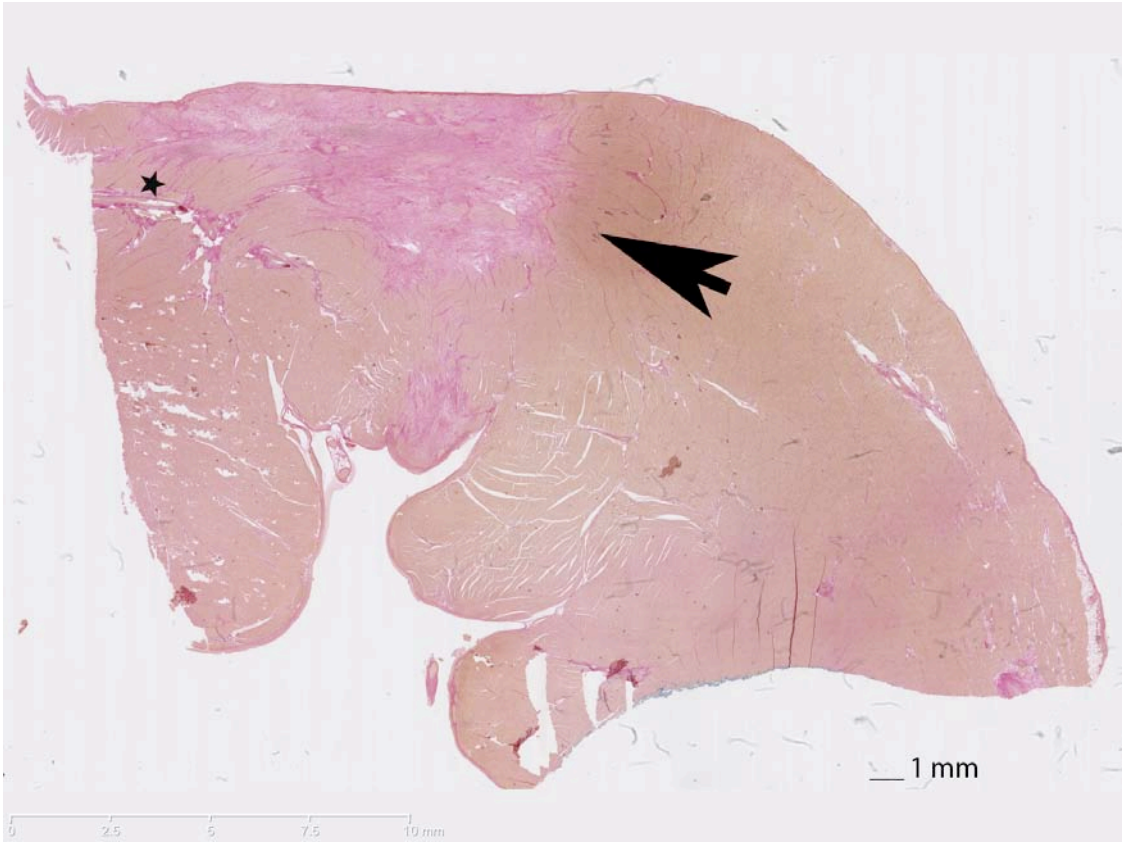


Figure 3.2.9. Van Giesson stained segment of LV myocardium from a placebo sheep showing a focus of replacement fibrosis (arrow) extending from the sub-epicardium. Striae of interstitial fibrosis are also evident to the left of the replacement fibrosis area (asterisk).

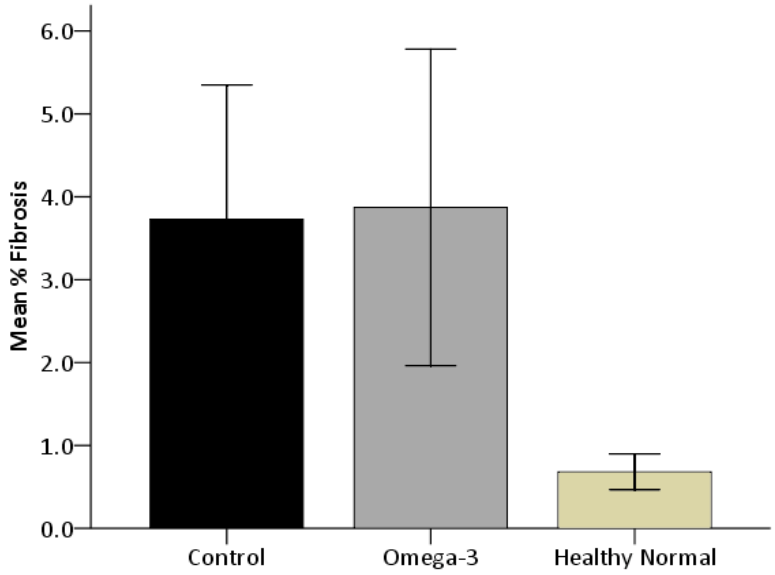


Figure 3.2.10A. Mean % area of total ventricular fibrosis for the placebo (n=7), omega-3 fed (n=6), and healthy normal (n=4) groups. Fibrosis burden was increased to a similar extent in both doxorubicin treated groups compared with untreated sheep ($p<0.05$).

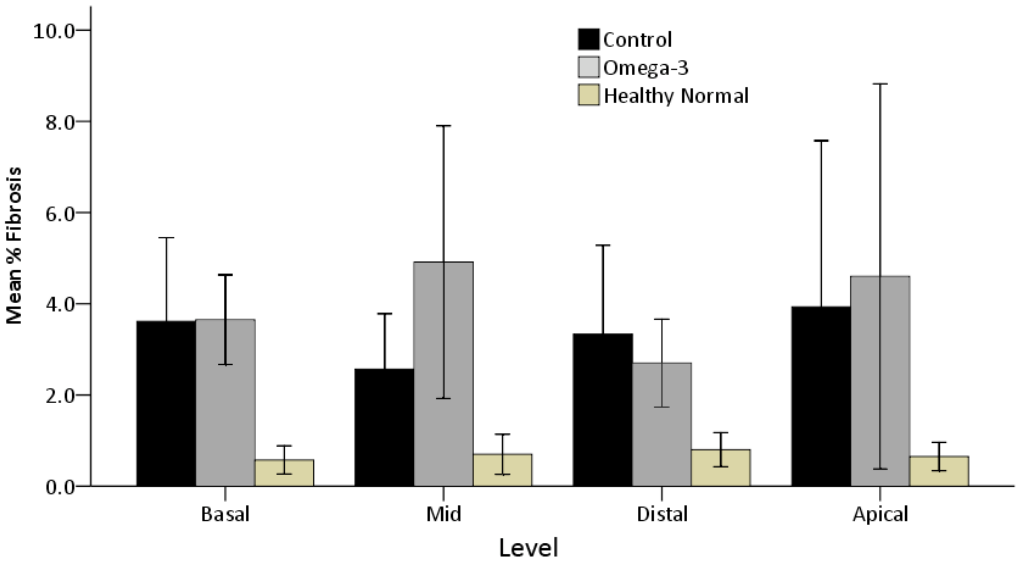


Figure 3.2.10B. Mean % area of fibrosis is shown for different myocardial levels sectioned transversely from ventricular myocardium. Fibrosis content was increased in both omega-3 and placebo group sheep at all levels of the myocardium, relative to the healthy controls. There was no significant segmental heterogeneity of fibrosis in either doxorubicin-treated group.

Discussion

4.1 Study Objectives

Despite a number of treatment regimens devised to mitigate the adverse cardiovascular effects of anthracyclines, chemotherapy-induced cardiomyopathy remains an important cause of patient morbidity and mortality. Recently, omega-3 PUFA have been considered for their adjunctive protective actions against anthracycline cardiotoxicity. Previous studies utilising either small animal or *in vitro* model systems have yielded conflicting findings, such that further translational research is required before progression to clinical investigation. We therefore conducted a novel, large animal study to evaluate the effects of dietary omega-3 PUFA supplementation on cardiac function and remodelling in a validated model of anthracycline-induced cardiomyopathy. This provided further translational preclinical data to complement the pre-existing basic scientific literature. The study utilised CMR, which is considered the current "gold standard" imaging modality for non-invasive assessment of cardiac volumes and function and state of the art histological analysis to quantify and compare levels of myocardial fibrosis between groups.

4.2 Uptake of Omega-3 PUFA in Merino Sheep

This study demonstrated that dietary supplementation of fish oil containing omega-3 PUFA in Merino wethers caused an elevation in erythrocyte and myocardial-bound levels of long chain omega-3 PUFA. An accompanying and significant reduction in the myocardial levels of arachidonic acid (ARA) in the myocardium in the omega-3 group sheep compared with controls ($p<0.05$), was also observed, suggesting that the

long chain omega-3 PUFA displaced phospholipid-bound ARA within cell membrane phospholipids. As indicated previously, incorporation of omega-3 fatty acids into the membrane phospholipid bilayer is a prerequisite for its biological activity[87]. These findings confirm that dehydrogenation of omega-3 PUFA in the sheep rumen following oral ingestion did not preclude its uptake into plasma and ultimately, phospholipid bound cell membranes. This has not been previously reported in the Merino sheep and demonstrates that this strain is a suitable preclinical large animal surrogate for multi-disciplinary studies assessing effects of dietary omega-3 PUFA supplementation.

4.2.1 Elevated Omega-3 PUFA levels at baseline

At baseline, the plasma and phospholipid bound levels of total omega-3 long chain PUFA were significantly higher in the dosing study sheep assigned to the control group compared with the omega-3 group. During the weeks leading up to the dosing study, all sheep were housed in large outdoor field pens. They received standard feed, lucerne chaff, however being housed outdoors, may also have had varying access to fresh pasture feed. Lucerne chaff contains negligible omega-3 PUFA, however fresh pasture feed may have contained sufficient levels of omega-3 PUFA to elevate the plasma and erythrocyte bound omega-3 PUFA levels in the control animals at baseline.

Changes were implemented following the initial dosing study in an attempt to address baseline variations in plasma and erythrocyte phospholipid bound omega-3 PUFA levels. These included transferring the animals from outdoor to indoor pen enclosures two weeks prior to the commencement of drenching, thereby removing all access to fresh pasture feed. However, some study animals in the second main study batch

nevertheless returned elevated baseline omega-3 levels. It is likely that levels of circulating and tissue bound omega-3 PUFA persisted in these sheep, even following two weeks abstinence from fresh pasture feed. Significantly however, omega-3 PUFA levels in all sheep receiving dietary supplementation of fish oil increased above baseline levels within 2-3 weeks of commencement of drenching, and stabilised to approximately two- to three-fold that of placebo group animals.

4.2.2 Implementation of Olive Oil placebo drenching for main study

The fish oil drenching protocol resulted in sheep receiving a high oil dietary intake compared with their normal diet, the effects of which were unknown in ruminant animals. The olive-oil placebo drenching was therefore implemented for the main study animals to account for any adverse or other systemic effects relating solely to a high oil intake diet.

4.3 Cardiac effect of Omega-3 PUFA in Ovine model of DOX-induced NICM

Dietary supplementation of omega-3 PUFA was associated with a greater decline in LV EF as assessed by CMR in response to intra-coronary anthracycline infusion compared with control animals receiving olive oil placebo. Furthermore, there was a significant increase in ESV and EDV as measured by CMR in the omega-3 group compared with placebo controls. This indicates that omega-3 PUFA resulted in augmented cardiac remodelling and systolic dysfunction in the setting of DOX exposure, a finding that rejects our primary hypothesis and contradicts the many other purported benefits of omega-3 PUFA on cardiac health. Notably, the similar levels of

fibrosis observed in both the control and omega-3 PUFA treated animals suggest that the greater adverse effect of DOX on cardiac function in the omega-3 group may be mediated by mechanism/s independent of fibrosis.

4.3.1 Possible mechanisms for adverse effects of Omega-3 PUFA on DOX-induced NICM

Administration of DOX induces cyclooxygenase-2 (COX-2) expression[118], which catalyzes the first step in the conversion of ARA to Prostglandins (PG). Prostaglandins are a group of 20 carbon lipid compounds synthesized ubiquitously within nucleated cells in response to injury. Though they are generally regarded as mediators of several pro-inflammatory effects, certain types such as PGE_1 and prostacyclin (PGI_2) also confer tissue benefits following injury, including suppression of apoptosis[119], suppression of necrosis and release of cytotoxic factors[120], and stimulation of neutrophil activation by dilating vasculature, particularly in disease conditions mediated by ROS[121].

In the rat model of cardiac injury by Dowd[119], DOX administered to rat neonatal cardiomyocytes induced cardiac cell injury, as demonstrated by an increase in plasma cardiac troponin-T, serum lactate dehydrogenase (LDH) and apoptosis. Blocking the release of PG via a specific COX-2 inhibitor (SC236) further aggravated the DOX-induced injury. In contrast, prior administration of a PG analogue (iloprost) attenuated the degree of injury. Thus, the release of PG by cardiomyocytes may have attenuated their sensitivity to DOX insult, largely via a reduction in apoptosis.

As discussed previously, incorporation of EPA and DHA into cell membranes occurs via substitution of ARA. In our study we observed a 30% reduction in myocardial bound ARA levels in the omega-3 PUFA fed animals compared with the placebo

group. EPA is an n-3 homologue of ARA and as such, it competitively inhibits COX-2 metabolism of ARA to PG. It acts as a competitive inhibitor. This reduction of COX-2-generated PGs may partly account for the mechanism by which omega-3 PUFA supplementation aggravated cardiotoxicity.

The cellular pathways which mediate anthracycline-induced cardiotoxicity are multiple and complex. The finding of similar fibrosis levels between the omega-3 and control groups in the current study may suggest that the greater decline in LV EF in the omega-3 group animals compared with control animals, was mediated by pathological pathways independent of fibrosis. As discussed previously, anthracyclines also induce apoptosis, down regulate cardiomyocyte contractile proteins and effect other changes leading to cardiomyopathy. One or more of these processes may be amplified in the context of omega-3 enrichment, which also exerts numerous cellular changes, manifesting greater decline in cardiac function.

Further assessment of histological markers such as apoptosis, contractile protein status, CMR parameters (including diastology and wall thickness changes between groups), as well as RNA micro-array analyses of myocardial samples are planned as a follow-on to this thesis to provide alternative mechanistic insights.

4.4 Attrition Rate

The attrition rate in this study was higher than anticipated. Six of 17 (35%) sheep died prematurely, compared with our previously observed mortality rate of 20%[109]. One reason for this difference in mortality rate may have been a subtle change to the DOX dosing regimen. Our previous protocol consisted of administering 3-4 doses of DOX at 0.75 and up to 1mg/kg, with a mean cumulative dose of 3.6 mg/kg[109]. In order to ensure consistent dosing amongst all animals in the current placebo-

controlled study, we opted to standardise the regimen to three doses of 1.2mg/kg. This also enabled an ethically favourable reduction in the number of procedural interventions, whilst achieving our optimal cumulative DOX dose to induce moderate and reproducible cardiomyopathy. Although there was some individual variation in response to doxorubicin, all sheep in the current study showed deterioration in global LV function by 12 weeks follow up. This follow-up period was longer than the six-week duration used in our validation study[109]. Indeed, two animals (one from each group) died from suspected cardiac arrhythmia between six and twelve weeks post-DOX, which also contributed to the high mortality rate observed in the current study. Anthracycline-induced cardiomyopathy was characterised in this study by moderate decline of LV EF and FS from baseline levels, and increases in EDV and ESV (omega-3 group only) as measured by CMR. It was also characterised by mild elevations in Troponin-T levels, accompanying changes in WCC and platelet levels, and significant myocardial fibrosis.

4.5 Study Limitations

4.5.1 Anthracycline Administration and Dosage

This study utilised intra-coronary administration of anthracyclines, with the objective of minimising systemic toxicity, and associated attrition rate, by achieving higher first pass concentration of DOX in the heart. In the clinical setting, chemotherapy agents are typically administered intra-venously. However in animal models of IV administered anthracycline, attrition rates of greater than 50% have been reported[122]. The model's strength is that it enables reproducible cardiotoxicity with acceptable animal mortality and overt morbidity. However, its limitation, is that it does not simulate the systemic administration of DOX in clinical chemotherapeutic practice.

Myocardial uptake of DOX might be less predictable by the IV route, although the difference between the two delivery strategies is unknown. Changes in somatic effects of DOX by adopting the intra-coronary compared with IV route are also unknown.

4.5.2 Absence of Neoplasia

The animals included in this study were young adults free of any known neoplastic or other adverse health conditions. The influence of neoplastic disease in this model is therefore unknown, including the pharmacological effects of DOX-infusion on tumour cells during dietary supplementation of omega-3 PUFA. Since the proposed model excluded neoplasia, the findings relate specifically to the effects on the myocardium of Omega-3 PUFA in sheep receiving DOX.

4.5.3 General Anaesthesia

All CMR and TTE measurements were performed under general anaesthesia. Though the anaesthetic protocol was standardised for each procedure throughout the study, the influence of anaesthesia on the cardiac outcomes assessed is unknown.

4.5.4 Follow-up Period

The 12 week follow up period in this study was selected to allow sufficient progression of DOX-induced NICM. It is believed that a longer follow up period in this model may have resulted in advancing disease progression and further escalation of the study attrition rate. In the clinical setting, patients may continue dietary supplementation of omega-3 PUFA for extended periods following chemotherapy.

The affects of long term omega-3 PUFA supplementation in the context of anthracycline-induced NICM is unknown.

4.5.5 Non reporting of some histopathology samples

Mean % area ventricular fibrosis were not reported for one of the omega-3 and two of the control group sheep due to quarantine restrictions placed at the IMVS field station at LARIF prohibiting the removal of their samples for omega-3 PUFA and fibrosis level analysis following the scheduled euthanasia. However, given the high level of variance of these levels between the placebo and omega-3 group sheep in the samples analysed, it is unlikely that the inclusion of myocardial and blood samples from the one additional placebo and two omega-3 group sheep hearts would have altered the outcome reported, which showed no statistically significant difference between these groups.

Summary and Future Directions

NICM contributes to approximately one third of cases of clinical heart failure and comprises various underlying aetiologies, including idiopathic, genetic, infectious, metabolic, iatrogenic and toxic processes[123].

In an ovine model of DOX-induced NICM, we found no beneficial effects of dietary supplementation with fish oil derived omega-3 long chain PUFA. This is despite confirming incorporation of omega-3 PUFA into the myocardial tissue. In fact, we found evidence of a detrimental effect with this therapy. Therefore, based on findings from this model, we would not recommend co-administration of omega-3 PUFA for the prevention of anthracycline-induced myocardial injury.

Basic mechanistic studies to better define the interactions between fatty acids and cardiac biology in the presence of anthracycline exposure are required before clinical evaluation can take place. This should include gene expression microarray, biomarker and histological analyses to further explore response to anthracycline and omega-3 PUFA co-administration, and with particular attention to inflammatory, oxidative stress and apoptotic pathways. Further, sub cellular structural changes should be evaluated to determine whether omega-3 PUFA attenuates or in fact potentiates these adverse effects.

The ovine model of DOX-induced cardiomyopathy described in this study has many potential applications for the investigation of novel therapies in the management of NICM, and has distinct advantages over small animal and in vitro models of DOX-induced cardiotoxicity.

Though this study was undertaken essentially in the context of cancer patients receiving anthracyclines, the model has proved particularly useful in investigating novel therapies in the setting of non-ischaemic heart disease.

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Appendix

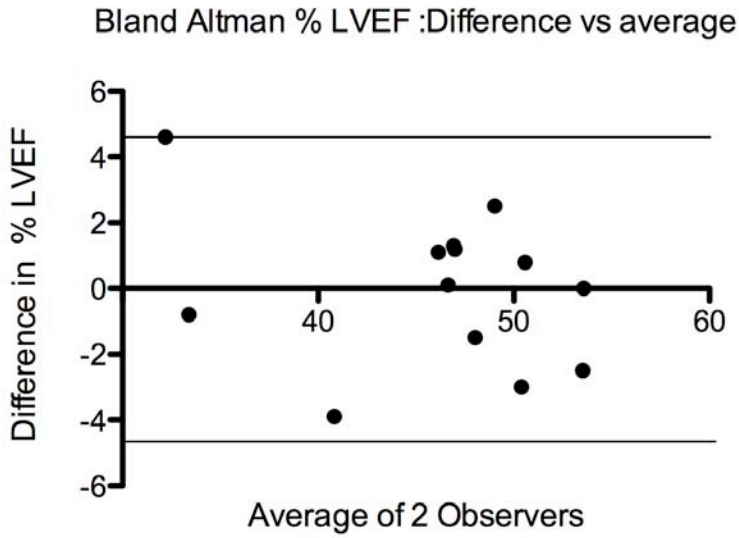
Reliability and precision of LV EF and % Area Fibrosis assessment.

For the LV EF and % area fibrosis analyses, the correlation coefficient between two observers was 0.68 and 0.92 respectively.

Bland Altman Bias and Agreement

	LV EF Analysis	% Area Fibrosis Analysis
Bias	0.008	0.31
SD of Bias	2.34	1.4
95% Limits of Agreement	±4.59%	±2.74%
From	-4.60	-2.5
To	4.58	3.1

The Bland Altman 95% limits of agreement were for LVEF (ie: SD of bias (2.34) x **1.96**), and ±2.74% for % area fibrosis (1.4 x **1.96**).



Bland Altman Mean % Area Fibrosis - Difference vs average