DIETARY FATTY ACIDS
AND THE HEART

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the degree of Doctor of Philosophy

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Department of Physiology
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Abstract

This Thesis is presented in the form of a compilation comprising 3 peer-reviewed research papers. Each paper is abstracted as below.

Publication #1

‘High quality RNA is the key to producing meaningful gene expression analyses. Human cardiac tissue specimens are extremely valuable, but may not always be obtained under optimal conditions and are frequently fibrotic. We provide a practical guide to assist in assessing the efficacy of two different RNA extraction methods applied to these challenging specimens. We describe how to compare ‘single-step’ and ‘multi-step’ extraction processes and discuss how to interpret information available through microfluidic and spectroscopic analyses to evaluate sample quality.’

Publication #2
Ip WTK, Chandramouli C, Smith JA, McLennan PL, Pepe S, Delbridge LMD. A Small Cohort Omega-3 PUFA Supplement Study: Implications of Stratifying According to Lipid Membrane Incorporation in Cardiac Surgical Patients. Heart Lung and Circulation resubmitted 28 October 2016 (acceptance pending)

‘Epidemiological studies and randomised clinical trials (RCTs) report disparate findings in relation to omega-3 polyunsaturated fatty acids (n-3 PUFA) benefit for cardiac patients. With RCTs interpretation is potentially confounded by background n-3 PUFA intake. The goal of this pilot, small cohort, pre-surgical supplementation study was to evaluate post-
operative atrial fibrillation (AF) and cardiac molecular expression profiles employing two data analysis approaches – by treatment randomisation and by stratification using measured n-3 PUFA. Patients (n = 20) received 3 grams / day of fish or placebo oil (FO vs PO) in a double blind randomised protocol prior to elective coronary artery graft and valve surgery. Groups were matched for age, gender, and mean treatment duration (~20 days). Resected atrial myocardium was sampled for assay of viability metabolic markers, and blood obtained for erythrocyte membrane lipid measurement. There was substantial overlap of cell membrane n-3 PUFA content across PO and FO groups, and no group treatment effects on AF incidence or myocardial molecular marker levels were detected. In contrast, data stratification using membrane n-3 PUFA content (at 8% total membrane lipid) achieved significant separation of patients (by n-6:n-3 PUFA ratio), a significant differential cardiac expression of the marker peroxisomal proliferator-activated receptor, but no difference in AF incidence. This small n-3 PUFA case study demonstrates that the same cohort may yield differing findings when evaluated using randomisation or stratification approaches based on direct molecular measures in cell membranes.

**Publication #3**


‘A definitive understanding of the role of dietary lipids in determining cardio-protection (or cardio-detriment) has been elusive. Randomized trial findings have been variable and sex-specificity of dietary interventions has not been determined. In this investigation the sex-selective cardiac functional effects of three diets enriched by omega-3 or omega-6 polyunsaturated fatty acids (PUFA) or enriched to an equivalent extent in saturated fatty acid components were examined in rats after an 8 week
treatment period. In females the myocardial membrane omega-6:omega-3 PUFA ratio was two-fold higher than male in the omega-6 diet replacement group. In diets specified to be high in omega-3 PUFA or in saturated fat, this sex difference was not apparent. Isolated cardiomyocyte and heart Langendorff perfusion experiments were performed, and molecular measures of cell viability assessed. Under basal conditions the contractile performance of omega-6 fed female cardiomyocytes and hearts was reduced compared with males. Omega-6 fed females exhibited impaired systolic resilience after ischemic insult. This response was associated with increased post ischemia necrotic cell damage evaluated by coronary lactate dehydrogenase during reperfusion in omega-6 fed females. Cardiac and myocyte functional parameters were not different between omega-3 and saturated fat dietary groups and within these groups there were no discernible sex differences. Our data provide evidence at both cardiac and cardiomyocyte level that dietary saturated fatty acid intake replacement with an omega-6 (but not omega-3) enriched diet has selective adverse cardiac effect in females. This finding has potential relevance in relation to women, cardiac risk and dietary management.’
DECLARATION

This Thesis comprises my original work towards the degree of Doctor of Philosophy except where indicated in the Preface (Tables of author contributions).

Wendy Tan Kei Ip

Date: 13/12/16.
# AUTHOR CONTRIBUTION

## Publication #1


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<td>• Methodology training: CEH</td>
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CEH, Catherine E Huggins; LMD, Lea MD Delbridge; SP, Salvatore Pepe; WTKI, Wendy TK Ip
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PUBLICATION #2

Ip WTK, Chandramouli C, Smith JA, McLennan PL, Pepe S, Delbridge LMD. A Small Cohort Omega-3 PUFA Supplement Study: Implications of Stratifying According to Lipid Membrane Incorporation in Cardiac Surgical Patients. Heart Lung and Circulation resubmitted 28 October 2016 (acceptance pending)

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<td>• Final manuscript review: <strong>WTKI</strong>, LMD, SP, PLM</td>
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CC, Chanchal Chandramouli; JAS, Julian A Smith; LMD, Lea MD Delbridge; PLM, Peter L McLennan; SP, Salvatore Pepe; WTKI, Wendy TK Ip
AUTHOR CONTRIBUTION

PUBLICATION #3


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ABBREVIATIONS

AF        atrial fibrillation
CHD       coronary heart disease
DART      Diet And Reinfarction Trial
FA        fatty acid
FO        fish oil
GISSI-HF  Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocarico-Heart Failure study
GISSI-P   Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocarico-Prevenzione study
JELIS     Japan EPA Lipid Intervention Study
LDH       lactate dehydrogenase
MUFA      monounsaturated fatty acid
n-3       Omega-3
n-6       Omega-6
N3D       Omega-3 intervention group
N6D       Omega-6 intervention group
NHFA      National Heart Foundation of Australia
ORIGIN    Outcome Reduction with an Initial Glargine Intervention trial
PGD$_3$   prostaglandin D$_3$
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<td>prostaglandin I₂</td>
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<td>ventricular fibrillation</td>
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# Abbreviations - Fatty Acids

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<th>Chemical Name</th>
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ADDITIONAL PUBLICATIONS

RELATED (NON-THESIS) PUBLICATIONS DURING CANDIDATURE:


CHAPTER 1

GENERAL INTRODUCTION

A potential general health benefit and a possible therapeutic role for preferential intake of polyunsaturated fatty acid (PUFA) type lipids has been a matter of public and practitioner interest for many decades. The earliest indications of PUFA benefit, specifically omega-3 class influence, arose from observations of population groups exhibiting apparently low incidence of cardiovascular disease associated with high level consumption of oily fish (enriched with omega-3 fatty acids) (Kromhout, Bosschieter, and de Lezenne Coulander 1985). Beyond cardiovascular and cardiac protection, in subsequent decades the concept of PUFA advantage, and specific omega-3 protection has been taken up in a myriad of health and disease settings – oncology, development, ageing, cognition, gastro-intestinal, respiratory, musculo-skeletal and more. As a ‘good fat’ the power of omega-3 has been supported by ‘functional food’ advocates, by proponents of ‘alternative therapies’ and by the nutraceutical industry.

The work presented in this Thesis, in the form of 3 integrated publications includes both clinical and pre-clinical original studies. Specifically, it relates to the pursuit of evidence of the efficacy of PUFA intake, in particular omega-3, as a therapeutic intervention relevant to cardiac disease. In providing a general introductory context, the commentary below outlines some of the basic chemistry of types and classes of dietary fatty acids, and provides an overview of the progression of the research over the past four decades. Finally, the rationale and overall goal of the studies comprising this Thesis is presented.
CHAPTER 2
LITERATURE REVIEW & PERSPECTIVE

2.1 DIETARY FATTY ACIDS TYPES, CLASSES AND NOMENCLATURE

A fatty acid is an organic molecule comprised of a long hydrocarbon chain and a carboxyl group (Figure 1). They are categorised into ‘types’ based on the degree of hydrocarbon chain saturation - i.e. number of carbon double bonds. There are three types of fatty acids: saturated fatty acids (SFA), with no carbon double bonds; monounsaturated fatty acids (MUFA) with one carbon double bond; and polyunsaturated fatty acids with two or more carbon double bonds. Fatty acid chain saturation determines rigidity of fatty acid structure. This is because carbon single bonds can only exist in one conformation – and hence exhibit more constrained spatial organization. Carbon double bonds can exist in either cis or trans conformation, conferring a more flexible structure.

Specific examples which illustrate these structural attributes are stearic acid (a common SFA of 18 carbon chain length) which is linear and rigid (Figure 1). Oleic acid (a common MUFA of 18 carbon chain length) has a cis-double bond introduced around the mid chain position and is more structurally constrained. Similar 18 carbon chain length PUFA s are linoleic and alpha-linolenic acid – each with differently positioned multiple double bonds and much more malleable with complex 3-dimensional configurations. The fatty acid nomenclature specifies carbon chain length, degree of saturation, and position of the first ‘unsaturated’ double bond relative to the methyl end of the carbon chain (designated as ‘o’ or ‘n’). Thus: stearic (18:0), oleic (18:1 n-9), linoleic (18:2 n-6) and alpha-linolenic acid (18:2 n-3). For MUFA and PUFA types the position of the first (or only) double bond designates fatty acid class: linoleic (18:2 n-6) is an omega-6 class and alpha-linolenic acid (18:2 n-3) an omega-3 class PUFA. Fatty
There are three types of fatty acids - saturated, monounsaturated and polyunsaturated fatty acids. They are categorized based on the number of carbon double bonds present, as illustrated by the double line in this figure. Here an example of each type of fatty acid is provided, with its chemical name and structure. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

**Figure 1 – Fatty acid structure and nomenclature.**
The polyunsaturated fatty acids that are of significance to human physiology and nutrition include the omega-3 (n-3) α-linolenic acid (ALA, 18:2 n-3), docosahexaenoic acid (DHA, 22:6 n-3), eicosapentaenoic acid (EPA, 20:5 n-3) and the omega-6 (n-6) PUFAs linoleic acid (LA, 18:2 n-6) and arachidonic acid (AA, 20:4 n-6). The n-3 ALA and the n-6 LA are generally described as ‘essential fatty acids’ because most mammals (including humans) are unable to synthesize them de novo and therefore must acquire them from diet. Essential fatty acids can be obtained from plant-based dietary sources such as flaxseed, walnut and canola oil. The longer chain PUFAs (e.g. EPA, DHA, AA) are not essential fatty acids, as they can be synthesized from ALA and LA via a series of desaturation and elongation processes (see Figure 2). However, it’s important to note that PUFA metabolism is inefficient – only <0.1 – 7.9% of ALA is converted to EPA, and <0.1 – 3.8% ALA is converted to DHA in humans (Plourde and Cunnane 2007). Therefore, recommended dietary intakes of EPA and DHA can only be achieved through ingestion of marine-derived lipids. Dietary sources of n-6 arachidonic acid (AA) include safflower oil and eggs; dietary n-3 PUFA docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can be obtained from marine sources - in particular oily fish such as salmon and tuna. Deriving from the link identified in early studies associating cardiovascular protection with oily fish dietary intake, the specific usage of DHA and EPA as supplements has been the focus of much investigation.

2.2 **Concepts of Omega-3 PUFA and Cellular Mechanisms**

It is understood that impact of fatty acid (FA) on cell structure and function arises from a range of different modes of action. Firstly, fatty acids can modulate membrane lipid-protein interaction. Membrane composition, as determined by the packing of FA types and classes together with cholesterol in the lipid bilayer can affect membrane ‘fluidity’. Fluidity status has direct impact on structure, mobility and function of membrane
Omega-6 fatty acids

18:2 n-6 (LA) → FADS2
18:3 n-6 → Elovl2
20:3 n-6 → FADS1
20:4 n-6 (AA) → Elovl2/5
22:4 n-6 → Elovl2
24:4 n-6 → FADS2
24:5 n-6 → Peroxisomal β-oxidation
22:6 n-6

Omega-3 fatty acids

18:3 n-3 (ALA) → FADS2
18:4 n-3 → Elovl2
20:3 n-3 → FADS1
20:5 n-3 (EPA) → Elovl2/5
22:5 n-3 → Elovl2
24:5 n-3 → FADS2
24:6 n-3 → Peroxisomal β-oxidation
22:6 n-3 (DHA)

Figure 2. Essential fatty acids and metabolism.

Long chain n-3 and n-6 PUFA can be synthesized from essential fatty acids linoleic acid (LA) and α-linolenic acid (ALA) via a series of elongation (addition of carbon atoms) or desaturations (addition of double bond) steps. Delta-6 desaturase (FADS2) is considered the rate limiting enzyme in the pathway. FADS2, delta-6 desaturase; FADS1, delta-5 desaturase; Elovl2, elongase-2; Elovl5, elongase-5.
bound proteins via mechanisms yet to be fully elucidated (Endo and Arita 2016). This includes transporters, exchangers and ion channels involved in excitation-contraction coupling in the heart such as the L-type voltage activated Ca\(^{2+}\) channel (Pepe et al. 1994) and Na\(^+\) channel (Xiao et al. 1995). Furthermore, the length and level of saturation of the fatty acids in the phospholipid is thought to affect the properties of cell membrane by altering the microdomain ‘rafts’ and ‘caveolae’ that function as signalling platforms. In vitro experiments have shown that n-3 PUFAs suppress protein kinase C signalling (Fan et al. 2004), and disrupt dimerization and recruitment of toll-like receptor 4 (Wong et al. 2009).

Fatty acids can be enzymatically extracted from cell membranes to serve as eicosanoid precursors (Salmon and Higgs 1987). Indeed, omega-3 (EPA and DHA) and omega-6 arachadonic acid (AA) are precursors to two different series of eicosanoids with different potencies on the activation of cellular inflammatory responses. The omega-6 series is generally regarded as pro-inflammatory (e.g. PGI\(_2\)), and the omega-3 series is more benign (e.g. PGD\(_3\)) (Tapiero et al. 2002). The cardiac beneficial effect of n-3 PUFA has been proposed to involve its anti-inflammatory action, by serving as alternate membrane substrate in eicosanoid production, resulting in the production of a less inflammatory profile (Endo and Arita 2016).

Fatty acids are also natural endogenous ligands to transcription factors and can modulate cellular signalling pathways by transcriptional regulation. A prominent example is the peroxisomal proliferator activated receptor (PPAR), which is known to play an important role in energy metabolism and survival regulation in numerous cell types, including cardiomyocytes (Azzouzi et al. 2011; Liu et al. 2015).

### 2.3 The beginning of the ‘Fish Story’ — epidemiological indicators

The story about the beneficial effects of marine-derived n-3 PUFAs on cardiovascular disease arose from observations of a lower incidence of coronary heart disease in populations with relatively high dietary consumption of fish (Bang and Dyerberg 1972).
At about the same time, the model of the ‘Mediterranean Diet’ emerged from studies showing that populations consuming diets rich in the omega-3 alpha-linolenic acid had reduced incidence of cardiovascular disease (Keys 1970). Furthermore, Mozaffarian et al showed that specific fish consumption pattern (tuna or other baked or broiled fish, but not fried fish) is associated with lower incidence of atrial fibrillation (Mozaffarian 2004). These findings were the basis for more than four decades of clinical and experimental studies that form the general paradigm of SFA detriment, PUFA benefit - with omega-3 specific PUFA advantage. Building on the initial observations, a number of subsequent studies showed strong association between fish intake and reduced cardiovascular mortality and/or events.

Extensive meta-analyses have been produced (Nestel et al. 2015; Del Gobbo et al. 2016), and two of the key primary studies reporting inverse relationship between fish intake and CHD outcomes are briefly noted here. The Diet and Reinfarction Trial (DART) was the first secondary prevention trial to assess n-3 effect in a group of post-MI men (Burr et al. 1989). Burr et al reported a significant improvement in survival rate in a patient cohort receiving dietary advice to increase fish intake, compared to a group that received no advice. Over a period of 2 years individuals were tracked, and the enhanced survival effect of fish advice was a 29% reduction in mortality. In another very large study involving 4,738 participants aged ≥ 65 years free of congestive heart failure at baseline, Mozaffarian et al reported a dose-dependent relationship between dietary fish intake (specifically tuna or other broiled or baked fish, but not fried fish) and reduction in heart-failure mortality (Mozaffarian et al. 2005). This is consistent with a meta-analysis by Zheng et al, which included 315,812 participants from 17 cohorts and an average follow-up period of 16 years. The authors concluded that there is a dose-dependent relationship between fish intake and relative risk of CHD death, and showed that every 15 grams / day increase of fish intake was associated with 6% decreased risk of CHD death (Zheng et al. 2012). Numerous other meta-analyses (but not all) have supported an beneficial effect of omega-3 on reducing cardiovascular events and deaths (Bucher et al. 2002; Hooper et al. 2004; Zhao et al. 2009; Mozaffarian and Wu 2011; Casula et al. 2013).
2.4 Experimental evidence of omega-3 cardiac action

In parallel with epidemiologic investigations, there has been considerable focus at the experimental level, discerning and defining in vitro and in vivo omega-3 specific actions. Much of this focus has been evaluation of cellular and cardiac responses associated with arrhythmogenesis, and some emphasis also on inotropic mechanisms. Extensive early in vitro work provided evidence that exogenous exposure to selected omega-3 PUFAs (compared with omega-6 PUFA or SFA) could elicit responses consistent with arrhythmia suppression (Kang and Leaf 1996; Kang and Leaf 2000). In neonatal cardiomyocytes, several different omega-3 molecular species were shown to promote more negative resting membrane potential and increase action potential trigger threshold level (Kang, Xiao, and Leaf 1995). In adult cardiomyocytes acute exposure to omega-3 PUFA was shown to inhibit pharmacological blockade and potentiation of L-type Ca\(^{2+}\) flux, implying a role for n-3 in the suppression of ischemia-reperfusion induced Ca\(^{2+}\) overload associated with arrhythmia (Pepe et al. 1994).

Experimental in vivo studies have also been numerous – also primarily targeted to identify anti-arrhythmic actions of omega-3 dietary intervention. Again, brief note is made of several key studies. In marmoset primates, fish oil feeding resulted in suppression of inducible ventricular fibrillation (VF) and elevation of VF threshold in vivo (McLennan et al. 1993; Charnock, McLennan, and Abeywardena 1992). With respect to omega-3 effect on cardiac contractile performance, Pepe and McLennan were among the earliest to show that dietary fish oil feeding resulted in enhanced ex vivo basal and post-ischemia reperfusion function associated with improved cardiac oxygen utilization efficiency (Pepe and McLennan 2002). At the in vitro level, cardiomyocytes isolated from rodents fed contrasting SFA, MUFA, and PUFA diets have been observed to exhibit different internal Ca\(^{2+}\) release dynamics, with an effect of fish-oil treatment consistent with arrhythmia suppression (Honen and Saint 2002). A more recent finding to emerge from the experimental literature has related to possible sex difference in responsiveness to dietary lipid intervention effects. In an omega-3 PUFA dietary feeding study reported by Huggins et al (2009), a beneficial functional effect of dietary omega-3 in augmenting post-ischemia recovery in a female rodent.
model was not apparent (Huggins et al. 2009). These finding suggest the possibility that n-3 PUFA may affect male and female differently.

### 2.5 Randomized controlled trials – the Fish Story gets fishy

The generally very positive findings arising from epidemiological work prompted the launch of numerous randomized controlled trials (RCTs) to evaluate PUFA (particularly omega-3) effect in primary and secondary prevention settings. The first n-3 PUFA RCT by Calo et al., tested the effects of n-3 PUFA supplementation on post-operative atrial fibrillation (AF) in coronary artery bypass graft (CABG) patients. The authors showed that n-3 supplementation for at least 5 days substantially reduced the occurrence of post-operative AF by 54%, and significantly shortened patient hospital stay (Calo et al. 2005). Later RCTs extended the n-3 supplementation duration and follow-up period, and explored additional cardiovascular outcomes. The Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocardico Prevenzione study (GISSI-P) testing omega-3 supplementation in a cohort of men (85%) and women (15%) after occurrence of myocardial infarction. Participants were given either fish oil (850mg EPA+DHA) or placebo capsules daily and were followed for a 4-year period. In this study, significant benefit of fish oil supplementation was found, including a 20% lower major cardiovascular event, 30% lower cardiovascular death and 45% lower sudden cardiac death (GISSI-investigators 1999). Similar omega-3 benefit was also reported in subsequent RCTs with patient cohorts of different disease background: the JELIS trial consisted of patients with hypercholesterolemia, and the GISSI-HF trial involved a patient cohort with moderate to severe heart failure symptoms (Yokoyama et al. 2007; Tavazzi et al. 2008).

However, omega-3 benefits have not been the consistent outcome in more recent RCTs (>2008). For example, the OMEGA trial which had a similar design to the GISSI-P study, consisted of a patient cohort with similar demographics, omega-3 treatment dose and endpoints, failed to identify treatment benefit (Rauch et al. 2010). Similarly, lack of benefit of omega-3 was also reported in the Outcome Reduction with an Initial
Glargine Intervention (ORIGIN) trial – one of the largest omega-3 RCTs conducted to-date (ORIGIN trial investigators et al. 2012). Indeed, with an overview covering over a four-decade period, the majority of meta-analyses have reported a finding of no effect of omega-3 PUFA (Hooper et al. 2004; Hooper et al. 2009; Khoueiry et al. 2013).

Thus, a considerable discrepancy in the findings reported in relation to omega-3 benefit using epidemiological and RCT approaches is apparent. This lack of clarity in relation to omega-3 effect has led to reconsideration of the recommendations offered by the National Heart Foundation of Australia (NHFA) in relation to omega-3 PUFA consumption in the form of supplementation in 2015. In 2008, NHFA recommended that for all adults free from coronary heart disease, a daily intake of 500 mg combined EPA and DHA (or 1000 mg for individuals with pre-existing heart disease) through combination of consumption of oily fish or omega-3 supplement (i.e. capsules). The recent review of evidence of omega-3 PUFA effect relating to prevention and treatment of cardiovascular disease, has produced a revised view regarding supplementation. This finding concluded that even though omega-3 intake from oily fish consumption has clear benefit, there is insufficient evidence to support the use of refined fish oil supplements in the context of coronary heart disease or atrial fibrillation (Nestel et al. 2015).

2.6 The Omega-3 PUFA effect controversy – Epidemiology & Observation vs RCTs

The inconsistency of the findings generated through observational studies and RCTs and omega-3 PUFA effect remains unresolved. It is important to recognize that in revisiting dietary recommendations regarding omega-3 intake, based on decades of findings from observation studies, the NHFA advice to continue to endorse fish intake was retained (Nestel et al. 2015), although benefit of supplementation was not. Whilst there may be a genuine difference in the effect of fish consumption versus omega-3 supplementation, it is also possible that the null findings in RCTs are due to inadequate
design and challenges associated with conducting nutritional research in human cohorts.

A ‘good’ RCT is one where exposure to the putative active agent is defined in both the ‘treatment’ and the ‘control’ groups. As has been recently highlighted (McLennan and Pepe 2015; James et al. 2014), in relation to omega-3 (or any dietary fatty acid) the strict management of this dichotomy is not possible because omega-3 PUFA (and other fatty acids) are always present to variable extent in background diet. In the case of omega-3 supplementation, there is also easy uncontrolled retail access. Thus the effectiveness of randomization has the potential to be significantly undermined, and lack of consistency/certainty in RCT outcomes may primarily reflect these limitations.

It is relevant that Mozaffarian et al. (2006) showed that maximum omega-3 benefit occurred at fish oil dose 250 mg / day with no further benefit with increasing intake (Mozaffarian and Rimm 2006). Evaluation of omega-3 intake of ORIGIN study participants identified a range from 40 – 468 mg / day (ORIGIN trial investigators et al. 2012). Not only was this wide range not adjusted for, the dose level was also higher than the ‘plateau’ dose reported as the maximally effective omega-3 PUFA dose (Mozaffarian and Rimm 2006). In this context the lack of effect of an additional omega-3 supplement superimposed on an already high baseline omega-3 intake in the ORIGIN study was not surprising.

Recently, these limitations relating to the real ‘dose’ effects in RCT design studies with fatty acid supplements have been highlighted. The compelling case has been put forward that progress cannot be made in this field unless actual tissue lipid profile is assessed, instead of presuming that randomization has defined the treatment status (McLennan and Pepe 2015). Importantly, relatively non-invasive means have been demonstrated and validated to make this clinically possible using erythrocyte membrane fatty acid composition as a surrogate for myocardial membrane fatty acid composition (Schacky 2014). It has now been well established in human and animal models that correlation between erythrocyte and myocardial lipid levels (including
omega-3) is robust for a wide range of lipid intake levels (Metcalf et al. 2010; Owen et al. 2004).

Further difficulties arise in relation to dietary / supplementation fatty acid intervention studies concerning the definition of ‘control’ (or even contrast) treatment. A universal placebo lacking ‘biological effect’ does not exist. A commonly used placebo has been olive oil, which was once thought to be relatively benign - but is now known to have cardiovascular effects and anti-oxidative properties (Ghorbel et al. 2015; Bulotta et al. 2014). In addition, with the use of supplement preparations there may be issues of fatty acid oxidation not accounted for during the course of study progression (Albert et al. 2016). There may be circumstances where manufacturing processes are sub-optimal or storage conditions compromised and lipid agents may be relatively easily oxidized in manner which modifies biological effect.

A related challenge in the dietary intervention research field is the problem of managing diet construction, where it is not simply a process of adding or withdrawing a component. To manipulate one lipid type inevitably results in modification of the relative levels of all fatty acid types – and managing macro-nutritional and caloric intake is also critical. In human studies very little control can be exerted over comprehensive diet construction – and even while one lipid type may be targeted for supplement, another may be subject to undefined parallel manipulation. In the experimental setting there is opportunity to achieve much greater control, and rather than reliance on supplementation there is capacity to fabricate structured dietary replacement regimes (although surprisingly often ‘control’ diets are designated as undefined ‘standard chow’).

2.7 Rethinking the ‘SFA is bad, PUFA is good’ stereotype

As outlined above, for some time there has been an embedded general paradigm of SFA detriment, PUFA benefit and omega-3 specific PUFA advantage. New controversial findings (clinical and experimental) have emerged suggesting that dietary SFA may not be ‘bad’ (Williams and Salter 2016; O’Connell et al. 2013) and that dietary n-6 PUFA
may not be ‘good’ (Julianne Beam 2015; Ramsden et al. 2013). Whilst systematic evaluation of these new propositions in relation to SFA benefit and/or omega-6 detriment are difficult to test in a clinical supplementation setting, in an experimental design where animal replacement diets may be rigorously implemented there is more scope for definitive investigation. The studies undertaken and presented in this Thesis address both these issues of management of treatment group dichotomy in the clinical setting with a supplementation study, and the use of fabricated replacement diets in an animal experimental setting.

2.8 The knowledge gaps

As outlined above, the difficulty in defining whether and how PUFA cardiac benefit may be conferred (when substituted for SFA) and if omega-3 compared to omega-6 augmentation produces distinctive effect, requires both new clinical and experimental approaches. A potential role for sexually dimorphic effects of PUFA requires exploration. An understanding of how clinical treatment groups may be confounded in the context of a randomized supplementation study is required. At the experimental level a rigorous investigation of cardiac functional effects of highly contrasted SFA and PUFA (both omega-3 and omega-6) replacement diets is required to achieve mechanistic insight. These two research goals are addressed in the studies presented in this Thesis.

2.9 Research goals and study approaches

The overall research goal was to evaluate the effect of manipulating PUFA intake in experimental and clinical settings on cardiac molecular and functional performance – with particular focus on establishing actual associated modification of membrane lipid composition.
Two independent and separate studies were undertaken:

1) A clinical omega-3 supplementation study (Publication #1, #2).

The goal of this small cohort pre-cardiac surgery omega-3 supplementation study was to evaluate effectiveness of randomization in producing well-contrasted n-3 treatment groups. Clinical and experimental outcomes were analysed employing two approaches – by treatment randomization and by stratification based on membrane omega-3 levels. A validation study was implemented prior to commencement of the randomization study to establish the optimal methodology for human tissue handling and molecular analyses.

2) A rodent diet PUFA replacement study (Publication #3)

The goal of this investigation was to establish the selective cardiac functional effects of three diets enriched by omega-3 or omega-6 polyunsaturated fatty acids or enriched to an equivalent extent in saturated fatty acid components. Rats were exposed to an 8-week diet treatment, basal cardiac and cardiomyocyte function was assessed and response to an acute ischemic event ex vivo evaluated. The experimental design incorporated a comprehensive analysis of sex-selective effects.

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CHAPTER 3
PUBLICATION #1

‘EVALUATING RNA PREPARATION OPTIONS FOR ARCHIVED MYOCARDIAL BIOPSIES’

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Evaluating RNA Preparation Options for Archived Myocardial Biopsies

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High quality RNA is the key to producing meaningful gene expression analyses. Human cardiac tissue specimens are extremely valuable, but may not always be obtained under optimal conditions and are frequently fibrotic. We provide a practical guide to assist in assessing the efficacy of two different RNA extraction methods applied to these challenging specimens. We describe how to compare ‘single-step’ and ‘multi-step’ extraction processes and discuss how to interpret information available through microfluidic and spectroscopic analyses to evaluate sample quality.

Background

Real-time PCR (qRT-PCR) gene expression analysis is a valuable tool in the study of human cardiac pathophysiology. qRT-PCR has the potential to generate extensive gene expression data from very modest quantities of sample material [1]. This makes it a particularly useful approach for studies where sample source is limited. It is well established that the use of high quality RNA is critical for the production of meaningful and reproducible qRT-PCR gene expression results [2]. Human atrial myocardial tissue specimens (i.e. obtained as discarded tissue from on-pump coronary artery bypass graft surgery) are extremely valuable, but are frequently fibrotic and may not be retrieved under optimal conditions – both factors which may limit the quality of RNA recovered. Thus, it is imperative to identify optimal RNA preparation methods for such samples and to ensure stringent sample quality control steps are performed prior to experimental analysis. In this report our goal was to consider how to most effectively extract RNA from such challenging samples by directly comparing results obtained from tissues subjected to different processing procedures in parallel. We compared two methods of purified RNA production: a ‘multi-step’ organic extraction based method and a ‘one-step’ solid-phase extraction method.

RNA Extraction Options to Consider

The organic (multi-step) RNA extraction method involves the use of phenol and chloroform under acidic conditions to separate sample cellular components into three phases: an aqueous phase, an inter-phase and an organic phase which contain cellular RNA, DNA and protein respectively [3]. RNA remains exclusively in the aqueous phase and can be recovered by precipitation with alcohol. One of the limitations associated with the organic multi-step method is the risk of co-extraction of phenol from the organic phase and/or DNA from the inter-phase whilst removing the RNA-containing aqueous phase. The presence of DNA and phenol in the final RNA sample can compromise downstream enzymatic reactions including reverse transcription and PCR amplification and quantification [4]. It is therefore important to reduce potential contamination by including an additional DNA removing step (e.g. DNase digest), as well as a purification step (to remove the phenol) following organic RNA extraction [5].

Alternatively, RNA can be prepared by solid-phase (one-step) extraction using silica membrane spin-column kits. This extraction method is based on the tendency of nucleic acids to adsorb to the silica membrane as the sample is being forced through the filter by centrifugation in the presence of a denaturing salt [6]. This method can incorporate a DNase digest treatment prior to being filtered through the column so any carry-over of phenol and DNA debris is washed off prior to RNA recovery.

Previous studies have compared the quality of RNA extracted by the organic extraction method to those prepared using silica membrane spin-column [7,8]. Okello et al. have performed a systematic comparison of seven...
nucleic acid commercial kits that were based on either the organic or solid-phase extraction method described above [8]. They reported that for their formalin-fixed paraffin-embedded autopsy samples (unspecified tissue type), the solid-phase method recovered most amplifiable RNA with the least PCR inhibition. However, a limitation of this comparison (and other similar studies) has been that the RNA recovered using the organic extraction method was not fully processed post-extraction by using appropriate purification steps. To date no study has directly compared spin-column extracted samples to the equivalently treated and purified organic extracted samples. We detail how to make such a direct comparison, and how to evaluate the outcome with specific reference to myocardial tissue. Full protocol details are provided in the accompanying Online Supplement.

Determining Optimal RNA Preparation Method for Myocardial Tissue

Right atrial appendages collected from cardiac surgery patients were pulverised using mortar and pestle on liquid nitrogen and divided into two 100 mg portions for parallel extraction using either the multi-step or one-step method (Fig. 1). Atrial appendages used in this study were stored at −80 °C for up to five years prior to the commencement of this study (see Online Supplement for additional information about comparing samples subjected to short and longer term freezer archiving). RNA quality defined in terms of intactness and purity was assessed by microfluidic electrophoresis and by spectrophotometry [9]. The ribosomal subunit RNA 28S:18S ratio and RNA Integrity Number (RIN) are both used as measures of RNA intactness. Sample purity was additionally determined based on the relative absorption of nucleic acid (260 nm) to protein (280 nm) and phenol (230 nm) for detection of protein and phenol contamination (see Online Supplement for extended explanation regarding the interpretation of these RNA quality measures).

RNA quality analysis of the multi-step and one-step samples is shown in Table 1. Compared to the multi-step method, paired-samples prepared using the one-step method exhibited significantly higher 28S:18S ratio and RIN values, suggesting that the RNA recovered is more intact and pure. In terms of the levels of contamination in samples, the levels of protein (A
260
:A
280
) and phenol (A
260
:A
230
) contamination were low in all samples and were not different between the two groups. This was supported by additional investigation of RNA quality based on visual signs of degradation on the electropherogram (refer to Online Supplement).

To assess how the differences in RNA quality affect downstream gene amplification, we performed qRT-PCR and determined gene expression of a messenger RNA gene, glyceraldehyde 6-phosphate (GAPDH) [10]. As illustrated in Fig. 2, samples prepared using the multi-step method exhibited apparently reduced GAPDH gene expression (relative units: 1.06 ± 0.22 vs 1.73 ± 0.24 for multi-step and one-step samples; n = 8; p < 0.05). Whilst...
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studies have reported that extended sample handling can cause RNA degradation [9], our parallel assessment of sample quality following each post-recovery preparation step showed no significant changes in RNA quality (see Online Supplement). This suggests that the reduced RNA quality was not caused by the additional post-recovery preparation, but may be more likely attributed to the organic extraction method. Based on these findings, we have concluded the one-step method is the preferred RNA preparation method for qRT-PCR gene expression studies involving this specific sample set of human atrial appendages. Additionally for samples of this type, we have been able to determine that RNA preparation is not necessarily adversely impacted by extended freezer storage (comparing one year and five year duration storage, see Online Supplement).

Conclusion

Whilst the practical clinical limitations related to the quality and quantity of samples available for research are difficult to overcome, there are modifiable factors that can be optimised to maximise the quality of results generated from these valuable samples. Using three methods to assess RNA quality, we have established that the solid-phase, one-step RNA preparation method is more suitable for our human heart tissues compared to the organic, multi-step method. We have provided a practical description of how method suitability may be evaluated for other sample collections so that RNA preparation approach can be tailored according to sample characteristics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hlc.2011.01.023.

References

ONLINE SUPPLEMENT

Evaluating RNA Preparation Options for Archived Myocardial Biopsies

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This Supplement provides supporting methods detail relating to the approach for evaluating RNA preparation options for archived myocardial biopsies. Additional data relating to RNA quality assessments performed at successive steps of the multi-step preparation method and comparing samples archived for different freezer storage periods are incorporated. Commentary about how to interpret measures of RNA quality generated by spectrophotometric and microfluidic electrophoretic analyses is also included.

**Tissue Recovery and RNA Preparation**

Tissues were derived from patients presenting for elective coronary artery bypass grafting (CABG) and/or valve repair surgeries at the Alfred Hospital. During surgery, a small segment of right atrial appendage (0.5 – 1 g), routinely removed as part of the cardio-pulmonary bypass procedure, was collected in ice-cold Ringer’s solution and immediately snap frozen in liquid nitrogen. The frozen tissue segments were pulverized into a powder form using mortar and pestle and separated into two 100mg samples and stored at -80°C for later RNA extraction by the ‘multi-step’ method, or the ‘one-step’ method.

For the multi-step method, RNA was extracted using the Invitrogen TRIzol® Reagent (Catalogue number 15596-018). Isolated RNA was subsequently DNase-treated using the Invitrogen DNase I, amplification grade (Cat # 18068-015) and purified using the Qiagen MinElute Clean-up kit (Cat # 74204). For the one-step method, applied to the paired matching samples, the Qiagen RNeasy Fibrous Tissue Midi kit (Catalogue Number 75742) was used. All RNA extraction, DNase treatment and RNA purification were performed according to the manufacturer’s instructions.
**Statistical Analyses**

Data are expressed as mean ± standard error of the mean (SEM). Data were analysed using Wilcoxon Signed Ranked test except otherwise stated. Data were considered significant at p < 0.05. All statistical calculations were performed using the Prism 4 (GraphPad software, USA).

**RNA Quality Assessment Procedures**

RNA quality assessment is based on evaluation of sample integrity (i.e. intactness) and purity (i.e. extent of contamination). In our approach, several integrity measures generated from the microfluidic electrophoretic assay were considered. To evaluate sample purity spectrophotometric measurements were used, to assess protein and solvent contamination of the RNA extract.

RNA integrity. Sample assays using the Agilent RNA 6000 Nano LapChip® kit and the Agilent Bioanalyzer 2100 were performed at the Australian Genome Research Facility (Parkville, Victoria, Australia). An electropherogram is the output generated from the Agilent Bioanalyzer that depicts the relative quantity of the different sample components detected in electrophoresis [1]. The Agilent LapChip® contains a set of micro-channels that is used for separation of nucleic acid species based on size. As RNA is driven through the separating matrix of the LapChip® electrophoretically, fluorescence dye within the matrix binds to the RNA, and the bound fluorescence is measured as RNA species migrate past the detector. The arbitrary fluorescence units of signal detected are converted into ug/ul RNA by reference to a ladder sample - which is a set of nucleic acid species of known size and concentration run along with the samples. Thus, the electropherogram provides a plot of the relative abundance of all nucleic acid species of different size [2]. The general visual features of the electropherogram are informative, and it also provides the source data from which some specific integrity measures can be determined (see below).
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The electropherogram is characterized by a marker fragment, 5S region, fast-region, 18S fragment, inter-region, 28S fragment and the post-region (Figure S1) [1]. A marker containing 50 bp DNA is run in each assay to allow alignment of all samples and is shown as the first peak on an electropherogram. 18S and 28S are the two most abundantly expressed RNA species and are readily detected in varying quantity depending on sample degradation state. The 5S region reflects the presence of small RNA species such as transfer RNA (tRNA), 5S RNA, 5.8S RNA in eukaryotic samples. In this study, no signals were observed in the 5S region in any sample. This verified the sample purification step where small nucleotides of < 200 bp were removed as part of the procedure. The electropherogram can be further sectioned into the fast-region (between 5S and 18S), inter-region (between 18S and 28S) and post-region (after 28S).

High quality RNA is characterized by a low signal in the fast-, inter- and post-region; and the presence of two distinctive 18S and 28S peaks with 28S peak being twice the height of the 18S peak. In contrast, RNA degradation is characterized by a reduction in 28S peak size relative to 18S; an elevation of signal in the inter-region that spreads to the lower molecular weight region (i.e. fast-region, 18S fragment and 5S region). The abundance (i.e. fluorescence signal amplitude) of 18S and 28S fragments is dependent on the amount of RNA input. In cases where samples of different concentrations are compared, it is the ultimate goal to focus on the relative abundance of 28S and 18S rRNA as a measure of sample integrity.

Representative electropherograms of a pair of matched multi-step (red) and one-step (blue) samples are illustrated in Figure S2. The 28S peak (relative to 18S) of the multi-step sample was markedly reduced (as circled in red). In addition, regions on the electropherogram that correspond to degraded 28S and 18S debris (i.e. the ‘inter-’ and ‘fast-’ region) of the multi-step samples were elevated while the one-step sample showed low baseline in all regions of the electropherogram. These findings suggest that the multi-step method produced samples that exhibited lower RNA quality profile compared to the one-step samples.

The 28S:18S ratio is a measure of RNA integrity. In intact eukaryotic RNA samples, the ratio of 28S: 18S is estimated to be close to 2:1, and a reduction in the ratio can be
visualized and interpreted as RNA degradation [3]. It has been suggested that intact RNA has an 28S:18S ratio of >1.8, although this ratio has been to be a variable and not necessarily accurate measure of RNA quality [4, 5]. To our knowledge, this is the first study that has compared RNA preparation methods in parallel using paired human cardiac tissues. Our data showed that the human cardiac RNA samples prepared using the multi-step method exhibited similar 28S:18S ratios to those found in other non-cardiac human tissues, including placental, liver and colon samples [6, 7]. Interestingly, the 28S:18S ratio of the RNA prepared by the one-step method presented in this study exceeded a value of 2.0, which is generally understood to be the ratio ascribed to a fully intact sample. This ratio benchmark was originally established on the basis of samples prepared using the conventional organic based extraction method [8], and it may be that with solid-phase extraction higher values can be achieved for some tissues.

The RNA Integrity Number (RIN) is a parameter developed by Agilent to describe sample degradation state using a score from 1 (completely degraded) to 10 (most intact) [1]. The manufacturer indicates that the RIN algorithm takes into consideration the degradation states of the entire sample (not just 28S and 18S) as shown on the various parts of the electropherogram: size of 18S and 28S fragments; signal at fast-region, inter-region and post-region. It has been demonstrated in a numerous studies that RIN is a more accurate RNA quality measure compared to the 28S:18S ratio [9].

RNA Purity. Spectrophotometric analysis was performed on samples diluted 1:100 10mM Tris-HCl, pH 7.5 using Eppendorf Biophotometer™ and Eppendorf UVett ® cuvettes (Catalogue no. 95201005-1) to generate measures of sample absorbance ratios at specified wavelengths for protein (A260:A280) and phenol (A260:A230) contamination. Use of these well established wavelengths relies on the spectrophotometric absorbance of nucleic acid at 260nm, proteins at 280nm and phenol & other organic contaminants at 230nm. A_{260:280} ratio of ≥ 1.7 and A_{260:230} ratio of ≥ 1.8 indicates little protein and phenol contamination [10].
**Chapter 3**

**Tracking RNA Quality During Multi-step Preparation**

RNA quality at the end of each of the three stages of multi-step extraction was assessed to monitor changes in RNA quality following DNase treatment and sample purification. ‘Total RNA’ (i.e. organic extracted only), ‘DNase RNA’ (organic extraction + DNase digest) and ‘Purified’ (organic extraction + DNase digest + purification) RNA were found to exhibit similar 28S:18S and RIN suggesting no significant RNA degradation as a result of the additional sample handling (28S:18S ratio: 1.37 ± 0.03 vs 1.26 ± 0.10 vs 1.25 ± 0.06; RIN: 7.95 ± 0.12 vs 7.28 ± 0.53 vs 7.61 ± 0.48 for total RNA, DNase treated RNA and purified RNA, n = 8, p > 0.05). Protein contamination (A260:A280) was found to be relatively higher following DNase digest but was reduced to negligible levels following purification (A260:A280: 1.99 ± 0.01 vs 2.35 ± 0.16 vs 1.93 ± 0.17 for total RNA, DNase treated RNA and purified RNA, n = 8), ‘Total RNA’ and ‘DNase RNA’ samples were found to contain significantly higher levels of phenol (A260:A230) which was removed in the subsequent purification step (A260:A230: 0.99 ± 0.20 vs 0.95 ± 0.36 vs 1.80 ± 0.60, for total RNA, DNase treated RNA and purified RNA, n = 8). These results suggest that reduced RNA quality was not caused by the additional post-recovery preparation, but more likely to be related to the initial organic extraction method *per se*.

**QRT-PCR Procedures**

*Reverse Transcription.* To obtain cDNA templates for real-time PCR gene analysis, isolated RNA preparations were reverse transcribed in a thermo-cycler (M. J. Research, PTC-100™ programmable Thermal Controller) using the *Invitrogen* SuperScript™ III First Strand Synthesis System for RT-PCR (Catalogue Number 1808-051) as described in the manufacturer’s manual.

*Primers Design & Optimization.* Human GAPDH primers were purchased as part of a pre-optimized PCR assay kit (Qiagen QuantiTect® Primer Assays, Catalogue number QT01192646) where primer sequence was not specified by the manufacturer. Serial
cDNA dilutions were performed and 4 ng cDNA per PCR assay was found to be the optimal condition for human GAPDH gene amplification in the absence of primer dimers. Human 18S primers were designed using Primer Express® software v2.0 (Applied Biosystems, USA). The forward and reverse primer sequences for 18S were: forward 5’-tcgaggccctgtaattggaa-3’; reverse 5’-ccctccaatggatcctcgtt-3’. Optimization determined that a primer concentration combination of 150/150nM (Fw/Rv) at 5 ng/reaction provided appropriate conditions to amplify the 18S genes at the lowest Ct with no primer dimer formation.

**Real-Time PCR.** Corbett Research Rotor-Gene 3000 was used for real-time PCR gene amplification and data collection (Rotorgene software, version 6). PCR amplification of 18S was initiated with a hold at 50°C for 2 minutes following by another hold at 95°C for 2 minutes. This was followed by 40 amplification cycles with 15 sec at 95°C, 30 seconds at 60°C. The measurements were acquired at the end of the annealing phrase at 60°C. Melt started from 60-99°C, held for 60 seconds on the first step, and 5 seconds on the following steps. Human GAPDH was amplified according to supplier instructions. Each sample was assayed in replicate (duplicate GAPDH and triplicate 18S) along with water blanks and no template controls in each PCR run. Melt curve analysis was performed at the end of each run to ensure single gene amplification. Threshold was set manually at the exponential phase of the amplification above the background. GAPDH expression was calculated using the comparative Ct method as previously described [11] and were expressed as relative units normalized to a reference gene (18S) to control for differences in the multistage sample preparation process (including potential differences in reverse transcription efficiency) that could otherwise bias the result.

**Effects of Sample Archive Period on RNA Quality**

In developing our approach, we also incorporated consideration of the effects of a variable period of freezer archiving on sample quality. A common issue associated with clinical studies is the slow sample acquisition process that can result in samples being
compiled in the freezer for extended freezer storage prior to experimental analysis. Ribonucleases (RNases) are ubiquitously expressed, highly active enzymes that can rapidly degrade sample RNA. Studies have shown that sample RNase can limit RNA recovery from samples that have been stored for an extended period of time [12]. In addition, there is evidence suggesting that sample vulnerability to RNA degradation is related to tissue-specific endogenous RNase content, with brain tissues being the most resilient to degradation, and the RNase-rich tissues such as liver being the most vulnerable to degradation [7, 13]. Human brain tissues stored at -70°C for more than five years have been shown to be suitable for conventional PCR gene expression studies [14]. The storage status of heart tissues over an extended period has not previously been examined. To address this question, we constructed a small pilot analysis to compare samples stored at -80°C for up to five years ('archived') and for less than one year ('recent'). For this analysis, only the single-step extraction process (detailed above) was employed were extracted using the Qiagen RNeasy Fibrous Tissue Midi kit (Catalogue Number 75742). RNA quality was assessed and qRT-PCR gene amplification of GAPDH was performed to determine downstream effects on qRT-PCR gene amplification.

Analysis of RNA quality based on 28S:18S ratio and RIN showed no significant differences between the archived and recent samples (28S:18S ratio: 2.66 ± 0.27 vs 2.81 ± 0.29; RIN: 9.33 ± 0.42 vs 9.22 ± 0.22 for archived and recent samples; n = 3 / 5, p > 0.05, Mann-Whitney U Test). As illustrated in Figure S3 the archived and recent samples displayed similar electropherogram characteristics that are suggestive of highly intact RNA. Furthermore, comparison of downstream qRT-PCR amplification of GAPDH showed no differences in expression levels (Figure S4). These findings suggest that high quality RNA can be produced from human atrial appendages that have been stored at -80°C for five years when RNA is prepared by the one-step method.
References


12. Lee KH, McKenna MJ, Sewell WF, Ung F. Ribonucleases may limit recovery of ribonucleic acids from archival human temporal bones. Laryngoscope. 1997 Sep;107(9):1228-34.


Figure S1 Electropherogram Characteristics. See text for commentary of features.
**Figure S2** RNA quality assessment: superimposed electropherograms of a set of paired one-step (blue) and multi-step (red) samples.
**Figure S3** RNA quality assessment: superimposed electropherograms of an archived (blue) and a recent (red) samples.
Figure S4 Archived and recent sample GAPDH gene expression. Purified RNA prepared from human right atrial appendages stored at -80°C for five years (archived) and less than one year (recent) was compared (relative unit: 1.67 ± 0.07 vs 1.77 ± 0.34 (mean ± SEM, n = 3 / 5 for archived and recent group respectively; p > 0.05). Relative gene expression was calculated using the comparative Ct method as previously described [11] with 18S as the reference gene. Statistical analysis was performed using Mann-Whitney U test (Prism V4.0).
CHAPTER 4
PUBLICICATION #2

‘A SMALL COHORT OMEGA-3 PUFA SUPPLEMENT STUDY: IMPLICATIONS OF STRATIFYING ACCORDING TO LIPID MEMBRANE INCORPORATION IN CARDIAC SURGICAL PATIENTS’

Heart Lung and Circulation; accepted on 12 December 2016
A Small Cohort Omega-3 PUFA Supplement Study:
Implications of Stratifying According to Lipid Membrane
Incorporation in Cardiac Surgical Patients

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Running Head:
Omega-3 PUFA & Cardioprotection

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Abstract

Background
Epidemiological studies and randomised clinical trials (RCTs) report disparate findings in relation to omega-3 polyunsaturated fatty acids (n-3 PUFA) benefit for cardiac patients. With RCTs interpretation is potentially confounded by background n-3 PUFA intake. The goal of this pilot, small cohort, pre-surgical supplementation study was to evaluate post-operative atrial fibrillation (AF) and cardiac molecular expression profiles employing two data analysis approaches – by treatment randomisation and by stratification using measured n-3 PUFA.

Methods
Patients (n=20) received 3g/day of fish or placebo oil (FO vs PO) in a double blind randomised protocol prior to elective coronary artery graft and valve surgery. Groups were matched for age, gender, and treatment duration (~20 days). Resected atrial myocardium was sampled for assay of viability metabolic markers, and blood obtained for erythrocyte membrane lipid measurement.

Results
There was substantial overlap of cell membrane n-3 PUFA content across PO and FO groups, and no group treatment effects on AF incidence or myocardial molecular marker levels were detected. In contrast, data stratification using membrane n-3 PUFA content (at 8% total membrane lipid) achieved significant separation of patients (by n-6:n-3 PUFA ratio), a significant differential cardiac expression of the marker peroxisomal proliferator-activated receptor, but no difference in AF incidence.

Conclusions
This small n-3 PUFA case study demonstrates that the same cohort may yield differing findings when evaluated using randomisation or stratification approaches based on direct molecular measures in cell membranes.

Keywords
omega-3 polyunsaturated fatty acids, atrial fibrillation, peroxisomal proliferator-activated receptor, Bax, Bcl2
Introduction

Evidence of the benefits of dietary intake of omega-3 long chain polyunsaturated fatty acids (n-3 PUFA) first emerged from population studies which identified a potential link between cardiovascular protection and dietary intake of marine fish-derived lipids [1]. Over an extended period, subsequent observational studies also reported positive association between n-3 PUFA consumption and cardiac mortality, especially in relation to myocardial infarction and sudden cardiac death [2-4]. These investigations have extensively documented anti-arrhythmic effects of n-3 PUFA, delivered via fish diet or supplementation [5-8].

However, a number of studies investigating the effects of short-term n-3 PUFA supplementation (implemented 1-5 days prior to CABG or valvular surgery) have failed to demonstrate a benefit in suppression of post-operative arrhythmias for patients not exhibiting sinus dysrhythmia before surgery [9,10]. Data relating to prevention of recurrent arrhythmia with longer term post-surgical supplementation were equivocal [11,12]. Overall, the most recent meta-analyses available of post-operative atrial fibrillation (AF) outcomes have produced disreputant results, both reduction and lack of significant reduction in AF with n-3 PUFA supplementation has been reported [13,14].

Reflecting the lack of resolution in the outcomes of these, and other supplementation trials and studies related to cardiovascular endpoints, over the last few years major international professional bodies have re-examined advice provided in relation to n-3 PUFA intake. An important recent development has been reconsideration of the recommendations offered by the National Heart Foundation of Australia (NHFA) in relation to adult consumption of n-3 PUFA. A major review of the evidence base available relating to n-3 PUFA involvement in the prevention and treatment of cardiovascular disease since the previous publication of NHFA recommendations in 2008 has been completed [15] concluding that, whilst dietary consumption of fish has clear benefit, the case for use of refined fish oil supplements in the context of coronary heart disease or atrial fibrillation is not supported [15,16].

The disparity in observational and randomised clinical trial-based findings in relation to n-3 PUFA supplement benefit is perplexing. The key issue and challenge which has been highlighted in the post-
hoc consideration of this NHFA report on n-3 PUFA efficacy is the difficulty in defining participant groups in randomised trials (or in any study design type) which can be identified as dichotomous treatment categories [17,18]. In dealing with a ‘treatment’ agent which is present always to a variable extent in background diet and which is readily available in non-prescription, retail supermarket form, the effectiveness of randomisation may be compromised. The importance of establishing actual tissue lipid incorporation levels to assess real dichotomy of treatment groups and cardiac endpoints has been emphasized [17,18]. Given that important earlier work has established in humans that erythrocyte plasma membrane lipid composition is a high fidelity surrogate measure of myocardial membrane lipid composition, it is surprising how few studies seek to make the link between actual tissue PUFA levels and cardiac outcomes within and between study subgroups [19].

In this, small cohort, pilot study of patients undergoing elective cardiac surgery, double-blind randomised to receive either n-3 PUFA or placebo, we have explored the association between individual patient tissue lipid status, post-surgical arrhythmia and levels of selected cardiac molecular measures. Atrial expression of several markers known to be responsive to ischemic stresses, both chronic (characteristic of perfusion insufficiency), and acute (as induced in a surgical setting), were examined. Our observations confirm that supplementation effects may only be inferred from stratified patient endpoint data relating to lipid status, and that group comparisons constructed through randomisation have inherent fallibility in this setting of compromised patient compliance and variable dietary baseline state.
Chapter 4

Materials and Methods

Patient Recruitment

Patients undergoing elective coronary artery bypass graft (CABG) surgery and/or valve repair/replacement surgery at Monash Medical Centre (Melbourne Australia) were recruited and consented for study participation. The study was approved by the Monash University Human Research Ethics Committee. Exclusion criteria were diabetes diagnosis and absence of stable sinus rhythm at pre-admission check. Patient medication history included ACE inhibition and statin therapy. At pre-admission clinic, patients were randomised to receive either fish oil (FO) or placebo (PO) treatment, 3 capsules/day dispensed by the hospital pharmacy in a double-blinded manner, ongoing for the pre-surgical period. Fish oil capsules (NUMEGA, Clover Corporation, Melbourne Australia) contained 1g tuna fish oil (25% docosahexaenoic acid (DHA, 22:6 n-3), 12% eicosapentaenoic acid (EPA, 20:5 n-3). Placebo capsules were manufactured in parallel and comprised mainly monounsaturated oleic acid (1g Sunola oil). At surgery, right atrial appendage biopsy samples were collected and snap frozen for tissue molecular analysis. Venous blood was collected for analysis of erythrocyte membrane lipids. Evidence of post-operative AF was determined from ECG records obtained during hospitalization.

Erythrocyte membrane lipid analysis

Total non-fractionated membrane phospholipid fatty acids were extracted and quantified as previously described [20,21]. Briefly, membrane phospholipids were isolated by solid-phase extraction and fatty acids were methylated by heating with addition of methanol, toluene and acetyl chloride. Fatty acids methyl esters produced were analyzed using gas chromatography by flame ionization detection. Fatty acids were identified from fatty acid methyl ester standards and expressed as a percentage of total fatty acids.

Atrial myocardium molecular analyses

Frozen atrial myocardium were pulverised under liquid nitrogen. Approximately 50 mg tissue was used for immunoblot protein expression analysis. Where tissue sample size permitted, an additional
portion (20 – 50mg) was analysed for mRNA by real-time quantitative polymerase chain reaction (qRT-PCR).

Protein immunoblot analyses were performed as previously described [22]. Tissues were homogenized (10% w/v) in 100mM Tris-HCl buffer with 5mM EGTA and 5mM EDTA (Sigma Aldrich, USA) containing protease and phosphatase inhibitor cocktail (complete protease inhibitor cocktail, Catalogue # 04693159001, Roche; PhosphoSTOP, Catalogue # 04906837001, Roche) at 4°C. Tissue homogenate was diluted in 2x sodium dodecyl sulfate (SDS) sample buffer. Protein concentration was measured by a modified Lowry assay to determine equal protein (μg) loading into polyacrylamide gels. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a TurboBlot system (Bio-Rad, CA, USA) followed by primary and secondary antibody incubation. Primary antibodies were purchased from Cell Signaling: Bax (#2772), Bcl2 (#2876), p-Akt(Ser473) (#9271), Akt (#9272). HRP-conjugated secondary antibody and chemiluminescent reagent (ECL-Plus RPN2133) were purchased from Amersham GE Healthcare. Chemiluminescent signal was imaged and quantified using QuantityOne software (Bio-Rad, CA, USA). To verify equal protein loading controls, after imaging membranes were stained with Coomassie Brilliant Blue R-250 as per manufacturer’s instructions (Bio-Rad Catalogue # 161-0436), re-imaged in white light and quantified using Bio-Rad QuantityOne software. For blot images, contrast/brightness optimization was applied uniformly to preserve relative densitometric integrity and no non-linear imaging adjustments were made.

Optimized mRNA analyses were performed as previously validated for human biopsy specimens [23]. A silica-membrane based mRNA extraction method using the Qiagen RNeasy Fibrous Tissue midi kit (Cat # 75742), including proteinase k digest and DNase treatment with on-column purification was implemented. cDNA was prepared (Invitrogen SuperScript™ III First Strand Synthesis System, # 1808-051) using a thermo-cycler (M. J. Research, PTC-100™ programmable Thermal Controller) according to manufacturer’s instructions. Human Bax, Bcl2 and PPARα (peroxisomal proliferation-activated receptor) primers were purchased as pre-optimized PCR assay kit (Qiagen QuantiTect® Primer Assays, Catalogue # QT00031192, QT00025011, QT00017451) where primer sequences were
not specified by the manufacturer. Human 18S primers were designed using Primer Express®
software v2.0 (Applied Biosystems, USA). The primer sequences were: forward 5’-
tgagccctgtaatggaa-3’; reverse 5’-ccctcaattgctctgtt-3’. Real-time PCR gene amplification and
data acquisition was performed using Corbett Research Rotor-Gene 3000 (software, V6). Human
Bax, Bcl2 and PPAR were amplified according to supplier instructions. PCR processing steps for 18S
were: initiation at 50 °C for 2 min and 95 °C for 2 min; amplification of 40 cycles at 95 °C for 15 sec
and 60 °C for 30 sec; annealing at 60 °C. Melt commenced from 60-99 °C, 60 sec first step, and 5 sec
for following steps. All samples assayed in replicate with water blanks and no template controls.
Threshold was set manually at the exponential phase of the amplification above the background.
Target gene expression levels were analysed using the comparative Ct method as previously described
[24] and were specified as relative units normalized to a reference gene (18S).

Statistical analyses

Data are presented as mean ± SEM unless otherwise stated. Differences between groups were assessed
using Independent t-test (nominal data) and Pearson chi-square test (categorical data). Regression
analysis was performed by linear regression test. Differences were considered significant when
p<0.05. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL).
Chapter 4

Results

Patient PO and FO groups matched for baseline characteristics

The baseline characteristics of the 20 patients randomised to the Fish Oil (FO) or Placebo (PO) treatment groups are shown in Table 1. There were no differences in average patient age or gender proportion between FO and PO groups. The average supplementation duration prior to surgery was not different between the two groups (19 ± 5 and 21 ± 10 days PO and FO). There was also no significant difference in the proportion of patients exhibiting one or more episodes of atrial fibrillation (AF) in PO and FO groups (50% and 30% respectively).

Expression of cardiac molecular markers not different between FO and PO treatment groups

A role for fatty acids in transcriptional regulation has been observed in various settings. Comparing PO and FO treatment groups we sought evidence of n-3 PUFA-associated difference in the expression of key signalling intermediates known to modify myocardial viability and metabolic signalling in ischemic stress. Expression of the apoptosis regulating proteins Bax and Bcl2 at both mRNA and protein levels were similar in the PO and FO groups, evaluated separately and ratiometrically (Figure 1A-C, 1E-G). There were also no differences between PO and FO groups in relation to expression of PPARα (an energy sensor and regulator molecule) or phosphorylated Akt (a protective intermediate of the PI3-Kinase pathway activated by ischemia) (Figure 1D and 1H).

Further analysis was undertaken to determine if a lack of difference in gene expression markers between PO and FO groups could be related to confounding effects of patient age or treatment duration on capacity for membrane incorporation within each treatment group. As shown in Figure 2A, 2B, there was no correlation between treatment duration and erythrocyte membrane EPA+DHA or DHA content in either the PO or FO group. Treatment duration for most patients was <2 weeks. Within each treatment group there was no correlation between age and membrane EPA+DHA (Figure 2C, 2E) or DHA (Figure 2D, 2F). Most notably, this analysis revealed extensive overlap of the range of membrane EPA and DHA values measured for the PO and FO groups.
Correlating expression of cardiac molecular markers with membrane n-3 PUFA content: combining PO and FO measurements

The overlap of erythrocyte membrane n-3 PUFA content between PO and FO groups prompted a re-examination of the relationships between cardiac molecular marker expression levels measured for all samples, combining both groups. In addition to evaluation of EPA+DHA content, the levels of EPA and DHA were assessed separately (Figure 3). The mRNA ratio of Bcl2:Bax expression and PPARα mRNA expression were each negatively correlated with EPA+DHA and with DHA alone (but not EPA). No significant relationships were observed between protein marker levels and any measure of n-3 PUFA.

Applying an alternative data stratification approach to construct treatment groups

Recently, a cogent case has been argued to apply a target n-3 PUFA treatment standard in evaluating (and constructing) participant groups to maximize the potential of achieving endpoint outcomes in dietary and supplementation studies. An ‘Omega-3 index’ (EPA+DHA) exceeding 8% has been recommended as a threshold level for identification of n-3 PUFA endpoint effect [25]. This approach was applied to the present data set. In Figure 4, the EPA+DHA sum is shown for the randomised PO and FO groups (Figure 4A), and in Figure 4B two alternative groups are constructed on the basis of stratification at the level of 8% EPA+DHA. Remarkably this process located 44% of PO assigned patients into the ‘below threshold’ group and 20% of the PO patients into the ‘above threshold’ group. Accordingly an analysis of the membrane n-3:n-6 PUFA ratio indicated a significant difference between stratified groups, but not between randomised groups (Figure 4C).

Expression of cardiac molecular markers and AF occurrence in stratified treatment groups

Finally, the expression levels of cardiac molecular markers and occurrence of AF was re-evaluated in the treatment groups re-constructed by stratification at the level of 8% total erythrocyte membrane EPA+DHA (Figure 5). With high n-3 PUFA levels (ie ≥ 8%), a significant group reduction effect on PPARα mRNA expression was detected, which had not been evident in the PO vs FO treatment contrast (Figure 5A). For the Bcl2:Bax and for the pAkt:Akt protein ratios, stratification did not render any difference in outcome when compared with the PO vs FO group analysis (Figure 5B and
5C). In relation to AF, whilst a slightly larger incidence differential was apparent between the stratified groups compared with the randomised groups, this was not a significant finding.

Discussion

Analysis approach matters: stratification vs randomisation

Arising from the disparate findings generated by epidemiological studies and RCTs in relation to n-3 PUFA intake benefit for cardiac patients, the importance of investigations where n-3 PUFA status for each patient is measured (rather than presumed on basis of treatment randomisation) has been highlighted [17]. Here we report the findings of a small case study (n=20) in which patient endpoint tissue molecular measures and post-operative AF occurrences were evaluated, implementing group analyses either by randomisation or by stratification using patient erythrocyte membrane n-3 PUFA levels. Prior to cardiac surgery, patients were randomised to receive 3g/day of fish oil by capsule or placebo oil (ie FO vs PO). Substantial overlap in the level of membrane EPA and DHA in PO and FO groups was observed. Whilst significant correlations between cardiac tissue expression levels of molecular markers involved in myocardial viability and metabolic signalling in ischemia stress and n-3 PUFA content were observed in the pooled patient cohort, no PO or FO group treatment effects could be discerned. Stratification of patient data on the basis of membrane EPA+DHA level (using a threshold value of 8% total membrane lipid) was demonstrated to produce an effective and significant dichotomous separation of patients (by membrane n-6:n-3 ratio). A significant difference in PPAR expression level between stratified groups was determined. Stratification slightly enhanced the AF group differential compared to randomisation group values, but significant AF benefit of omega-3 status was not detectable in either analysis setting. Albeit a pilot study of modest dimension and scope, this investigation offers case evidence in relation to omega-3 efficacy, showing that the same cohort may present different characteristics and findings when evaluated from randomisation and from stratification perspectives.
Defining treatment groups in the context of variable omega-3 PUFA background and compliance challenge

The discrepant findings reported from population/observational studies and from RCTs in relation to n-3 PUFA benefit, at least partially reflect the problem of variable participant n-3 PUFA status at study commencement and ongoing ingestion behaviour during the study [15]. In supplementation studies, participants will have variable endogenous background levels of key PUFAs, and the capacity to effect a quantitatively significant intervention by supplementation may be compromised [17]. Evidence suggests that even relatively low dose n-3 PUFA (ie 250mg EPA+DHA/day) can influence cardiovascular outcome, a dose level easily achieved with a single meal dietary adjustment [25, 26]. An early case-control study, at a time before publicity of cardiac benefits of n-3 PUFA, established that erythrocyte EPA+DHA and dietary intake correlated, and both were inversely related to risk of primary cardiac arrest [8]. Today, study participants with concern of disadvantage conferred by placebo randomisation may self-treat very simply (through independent retail supplement access or dietary modification) or the variable and often short interval from treatment to surgery will lead to variable and inadequate tissue incorporation required for physiological effect, as demonstrated in animal studies [20, 21]. It has been proposed that an optimal RCT design to detect n-3 PUFA benefit requires selection of study participants with low background n-3 PUFA levels [25-27]. Whilst this approach might have practical/ethical challenge, at least the rigorous implementation of tissue measurement of n-3 PUFA levels at endpoint (and possibly at study entry) seems obligatory. Our membrane analysis data showed clearly that there was substantial overlap in membrane n-3 PUFA content in randomised FO and PO groups. Indeed when stratification was applied (at the erythrocyte 8% EPA+DHA threshold level proposed by von Schacky [25]), there was significant group transfer of participants: 20% of the PO group localized into the upper strata, and 44% of the FO participants to the lower strata. These observations suggest that in this particular cohort, supplement compliance and treatment duration posed more of a difficulty than dietary confounding. The use of the n-6:n-3 PUFA ratio provided particularly useful discrimination between patient groups, and this is consistent with previous clinical and experimental reports [10,28].
Myocardial molecular markers, AF and erythrocyte lipids

Although the primary clinical endpoint of interest in relation to n-3 PUFA supplementation efficacy was post-operative AF, the present exploratory study sample size was not designed to offer statistical power to contrast potential treatment effects on the binary incidence of post-operative AF. Not surprisingly neither randomisation nor stratification analysis approaches could identify an effect of n-3 PUFA status on AF. Aside from this limitation, notably, this lack of significant difference in the incidence of AF between group contrasts may also reflect the finding that even the lowest n-3 EPA+DHA content level recorded was ~ 6.6%. Recent meta-analysis data indicate that this is not a low value [25], and suggests that all study participants were already potentially amply ‘dosed’ with n-3 PUFA.

As secondary markers of myocardial n-3 PUFA signalling effects, expression levels of a few select molecules involved in viability signalling and metabolic regulation, particularly in the ischemic-stressed myocardium, were evaluated. The protein expression levels of the apoptosis regulating proteins Bax and Bcl2 were measured, and where tissue quantity allowed, mRNA levels were also assessed. The Bcl2:Bax ratio is generally understood to be an indication of propensity to limit apoptosis. The phosphorylation (ie activated, Ser 473) status of the PI3-kinase trophic signalling marker Akt (relative to total Akt) was measured. In addition the mRNA levels of peroxisomal proliferator-activated receptor (PPARs) were determined. PPAR is known to be responsive to endogenous long chain fatty acids (including n-3 PUFA) and has a role in metabolic signalling through transcriptional regulation of genes [29,30].

By evaluation of pooled data from all patients, negative correlations were found between the content of n-3 PUFA and PPAR levels (and Bcl2:Bax), primarily driven by DHA (compared to EPA). In the stratified data analysis, a significantly reduced PPAR level in the upper level strata (~ 8% total fatty acids) also persisted, whereas Bcl2:Bax findings were marginally not significant. The first conclusion is that these apoptotic expression responses appear to be ‘early indicators’ as the mRNA shifts were not coincident with protein shifts. Secondly, the finding of inverse correlation between n-3 PUFA membrane content and both PPAR and Bcl2:Bax mRNA ratio was suggestive of n-3 PUFA signalling...
feedback inhibition. Although not extensive, there is some experimental literature suggesting n-3 PUFA apoptosis suppression in oncogenic settings and neural cell types [31,32]. However as we did not directly investigate more definitive apoptotic measures, our findings in the limited context of this study cannot be considered mechanistically definitive but provide a basis for further discovery work in larger treatment groups in relation to n-3 transcriptional and translational regulation in the heart. Despite the limitations of measuring only a small number of ‘candidate’ genes and proteins, and the constrained group sizes which emerged after data stratification, these molecular markers provided utility for exploring secondary endpoint study methodology.

Conclusion

The major finding of the present study is the demonstration that, even in a small patient cohort, evaluation of n-3 supplementation efficacy by stratification methods based on tissue n-3 content levels has the capacity to generate a more decisive finding in relation to cardiac endpoint analysis than can be achieved through reliance on randomisation group analysis.

Acknowledgements

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Disclosures

None.
References


prescription omega-3 fatty acids for the prevention of recurrent symptomatic atrial fibrillation: a randomized controlled trial. JAMA 2010; 304: 2363-72.


Table 1 Patient details. AF, atrial fibrillation; M, male; F, female; Y, yes; N, no. Data are presented as mean ±SEM. Non-paired t-test for age and treatment period; Pearson Chi-square test (ie AF, gender).

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Mean (PO) 67 ± 3 (years) 80% M (8M + 2F) 19 ± 5 (days) 50% (5/10)

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Mean (FO) 61 ± 4 (years) 70% M (7M + 3F) 21 ± 10 (days) 30% (3/10)
Figures

**Figure 1.** Cardiac molecular markers in PO and FO groups.
A-D. mRNA expression (by qRT-PCR) of apoptotic proteins Bax and Bcl2, and metabolic transcriptional factor PPAR.
E-H. Protein expression (by immunoblot) of apoptotic proteins Bax and Bcl2, and phospho-activation of trophic mediator Akt (Ser 473) normalized to total Akt (pAkt:tAkt).
Data presented mean ± SEM. Non-paired t-test, p = n.s. PO, Placebo Oil; FO, Fish Oil.

**Figure 2.** A, B. Correlation of erythrocyte membrane EPA+DHA and DHA only (% total fatty acids) with patient treatment duration in PO and FO groups. C, D. Correlation of erythrocyte membrane EPA+DHA and DHA only (% total fatty acids) with patient age in PO group. E, F. Correlation of erythrocyte membrane EPA+DHA and DHA only (% total fatty acids) with patient age in FO group.
Data analysed by linear regression test. PO, Placebo Oil; FO, Fish Oil. Data analysed by linear regression test. PO, Placebo Oil; FO, Fish Oil.

**Figure 3.** A. Correlation of cardiac molecular markers with erythrocyte EPA content (pooled PO & FO groups). B. Correlation of cardiac molecular markers with erythrocyte DHA content (pooled PO & FO groups). C. Correlation of cardiac molecular markers with erythrocyte total EPA+DHA (pooled PO & FO groups). Data analysed by linear regression test. n = 11–16; *p<0.05. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.
Figure 4. Membrane EPA+DHA: groups by randomisation and stratification

A. Erythrocyte membrane EPA+DHA as % fatty acids for PO and FO randomized groups.

B. Erythrocyte membrane EPA+DHA as % fatty acids for < 8% and ≥ 8% stratified groups
   Data shown as dot plots with mean (horizontal bar), and threshold stratification level (8%) dashed line.

C. Erythrocyte membrane n-6:n-3 ratio for randomized and stratified groups.
   Data presented mean ± SEM. Non-paired t-test, *p < 0.05.

PO, Placebo Oil; FO, Fish Oil. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

Figure 5. Cardiac molecular markers and AF in randomised and stratified groups.

A. PPAR mRNA expression for randomised and stratified groups.

B. Bcl2:Bax protein expression ratio for randomised and stratified groups.

C. pAkt (ser 473):tAkt protein expression ratio for randomized and stratified groups.
   Data presented mean ± SEM. Non-paired t-test, *p< 0.05.

D. AF occurrence for randomised and stratified groups.
   For stratified groups membrane EPA+DHA as % fatty acids (< 8% ≥ 8% total fatty acids). Data analysed by Pearson Chi-square test p = n.s.

PO, Placebo Oil; FO, Fish Oil.
Figure 2

A. FO ($r^2 = 0.228, p = 0.163$) and PO ($r^2 = 0.002, p = 0.904$) relationship between membrane EPA+DHA and treatment duration (days).

B. FO ($r^2 = 0.328, p = 0.083$) and PO ($r^2 = 0.326, p = 0.085$) relationship between membrane DHA and treatment duration (days).

C. PO ($r^2 = 0.000, p = 0.993$) relationship between membrane EPA+DHA and age (years).

D. PO ($r^2 = 0.190, p = 0.208$) relationship between membrane DHA and age (years).

E. FO ($r^2 = 0.069, p = 0.462$) relationship between membrane EPA+DHA and age (years).

F. FO ($r^2 = 0.080, p = 0.430$) relationship between membrane DHA and age (years).
Figure 3

A  EPA

B  DHA

C  EPA+DHA
Figure 4

A  Membrane EPA+DHA

B  Membrane EPA+DHA

C  Membrane n-6:n-3
Figure 5

A. **PPAR (mRNA)**
- **ΔΔCt fold change (18S)**
- PO / <8% group
- FO / ≥8% group

B. **Bcl2:Bax (protein)**
- **Arb Unit**

C. **pAkt:tAkt (protein)**
- **Arb Unit**

D. **Atrial fibrillation**
- **% patient**
- 50% 33% 50% 29%
CHAPTER 5
PUBLICATION #3

‘DIETARY OMEGA-6 FATTY ACID REPLACEMENT SELECTIVELY IMPAIRS CARDIAC FUNCTIONAL RECOVERY AFTER ISCHEMIA IN FEMALE (BUT NOT MALE) RATS’

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Dietary omega-6 fatty acid replacement selectively impairs cardiac functional recovery after ischemia in female (but not male) rats

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Running head: Dietary omega-6 PUFA and impaired female cardioprotection

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Chapter 5

ABSTRACT

A definitive understanding of the role of dietary lipids in determining cardio-protection (or cardio-detriment) has been elusive. Randomized trial findings have been variable and sex-specificity of dietary interventions has not been determined. In this investigation the sex-selective cardiac functional effects of three diets enriched by omega-3 or omega-6 polyunsaturated fatty acids (PUFA) or enriched to an equivalent extent in saturated fatty acid components were examined in rats after an 8 week treatment period. In females the myocardial membrane omega-6:omega-3 PUFA ratio was two-fold higher than male in the omega-6 diet replacement group. In diets specified to be high in omega-3 PUFA or in saturated fat, this sex difference was not apparent. Isolated cardiomyocyte and heart Langendorff perfusion experiments were performed, and molecular measures of cell viability assessed. Under basal conditions the contractile performance of omega-6 fed female cardiomyocytes and hearts was reduced compared with males. Omega-6 fed females exhibited impaired systolic resilience after ischemic insult. This response was associated with increased post ischemia necrotic cell damage evaluated by coronary lactate dehydrogenase during reperfusion in omega-6 fed females. Cardiac and myocyte functional parameters were not different between omega-3 and saturated fat dietary groups and within these groups there were no discernible sex differences. Our data provide evidence at both cardiac and cardiomyocyte level that dietary saturated fatty acid intake replacement with an omega-6 (but not omega-3) enriched diet has selective adverse cardiac effect in females. This finding has potential relevance in relation to women, cardiac risk and dietary management.

Key Words:
dietary polyunsaturated fatty acids, ischemia-reperfusion, heart, cardiomyocyte, sex difference.
NEW AND NOTEWORTHY

Using stringently controlled diet treatments, this research provides novel evidence at both heart and cardiomyocyte level that dietary saturated fatty acid intake replacement with omega-6 (but not omega-3) enriched diet has selective adverse functional effect in females.

This finding has potential relevance relating to women, cardiac risk and dietary management.
INTRODUCTION

An understanding of the role of dietary lipids in determining cardio-protection (or cardio-detriment) has been elusive. More than three decades ago, population studies attributed reduced susceptibility to myocardial infarct to high dietary intake of omega-3 (n-3) fatty acids (14). Numerous observational studies have reported links between consumption of diets with high levels of polyunsaturated fatty acids (PUFA), n-3-rich in particular, and reduced cardiac mortality (11, 24). However, establishing selective PUFA benefit through randomized controlled trials has been difficult and controversial (19, 25, 46). The general paradigm pursued has been that increased PUFA intake (relative to intake of saturated fatty acids, SFA) confers benefit, and that long chain n-3 (docosahexaenoic, DHA and eicosapentaenoic acids, EPA) versus omega-6 (n-6) PUFA are of special advantage. Large trials and meta-analyses have produced inconsistent/inconclusive findings and a number of reasons have been identified for failure to achieve definitive outcomes regarding cardioprotection (9, 26).

In relation to the question of n-3 benefit specifically, the difficulty in identifying a dietary reference control option or placebo supplement lipid has been problematic. In dietary trials, the background dietary context and compliance behaviour of trial participants limits the capacity to generate well contrasted and defined treatment groups. Experimentally, efforts to discern cardio-protective mechanistic insight in relation to lipid exposure have produced similarly equivocal findings. The use of undefined ‘standard chow’ diets as reference and the caloric/oxidative effects of using oil additives to shift diet lipid profiles have produced discrepant outcomes (3, 18). In the absence of an underlying pathology, short durations of dietary intervention (3-8 weeks) were found to have no effect on baseline cardiac contractile function measured in vivo and ex vivo (porcine & rodent) (13, 20), whereas chronic diet treatment (24 months) improved baseline ejection fraction in marmoset monkeys (31).

Conversely, two weeks of fish oil supplementation was sufficient to improve baseline
ejection fraction of mice with cardiomyopathy induced by systemic carnitine deficiency (45).

Cardiac contractile performance (ex vivo) following ischemia has been reported to be
improved or not changed in hearts of animals fed a fish oil supplemented diet compared to a
control diet (41, 42, 48). Ex vivo cardiac energetic studies indicate that oxygen consumption
in a setting of n-3 PUFA is more favourable in relation to work output post-ischemia (41) and
also in a setting of increased work demand associated with cardiac hypertrophy (30).

Interestingly, favourable energetic and functional indices have also been recently reported
with increased dietary SFA intake in a rodent experimental in vivo model of myocardial
infarction (7) and enhanced survival with SFA feeding in a model of failure (16). These
studies suggest that a more nuanced understanding of dietary lipid influence on cardiac and
cardiomyocyte function is required (44).

No direct experimental investigation of the sex selective actions of dietary lipids on cardiac
performance has been reported – despite an emerging clinical literature suggestive of gender-
related differences in response to lipid dietary intervention (47, 49). A single investigation
focussing on a female-only rodent cohort has suggested that omega-3 dietary intervention
may not produce benefit (21). Given the recent emphasis on achieving more extensive pre-
clinical characterization of sex-specific responses to potential therapeutic interventions (35),
the case for addressing this dietary lipid knowledge gap is compelling.

In this study, a sex-difference in cardiac functional responses to experimental dietary PUFA
and SFA replacement regimes was hypothesized. A comparative investigation of
performance of hearts and cardiomyocytes derived from female and male rats fed diets
specified to maximize contrast between dietary lipid type (ie SFA vs PUFA diet) and
between lipid class (n-6 vs n-3) across type-matched diets was undertaken. Three
macronutrient specified diets were designed, enriched in SFA (SFD), n-3 PUFA (N3D) or n-6
PUFA (N6D). Isolated cardiomyocyte electromechanical characteristics were evaluated and
the responses of isolated hearts to ischemia-reperfusion challenge assessed. Our findings
provide evidence at the myocardial and cellular level that an n-6 PUFA enriched diet
selectively influences cardiomyocyte performance and impairs functional recovery after
ischemia in females.
MATERIALS AND METHODS

Rodent diet design and fabrication

Experiments were conducted and animals handled in the manner specified by the NHMRC/CSIRO/ACC Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (1997), with approval and oversight by the University of Melbourne Animal Ethics Committee. Three rodent diets - n-3 diet (N3D), n-6 diet (N6D) and saturated fat diet (SFD), were designed to compare three distinct dietary fatty acid 'replacement' regimes. Diets were manufactured by Specialty Feeds (Perth, Western Australia, Cat #s SF06-094/095/096). Diet formulations were based on the AIN-93G standard chow rodent diet with lipid composition modified. The three isocaloric diets were fabricated, within macronutrient and source ingredient constraints to contain equivalent digestible energy (16.8 MJ/Kg) and comprising 10% fat, 24% protein, and 54% carbohydrate. MUFA was held constant at ~30% of total fat. For the SFD, the non-MUFA fatty acid content in the diet was enriched with saturated fat (ie cocoa butter) while PUFA was kept minimal (5% total n-3 PUFA and <0.5% n-6 PUFA). For the two PUFA diets - N3D and N6D, the non-MUFA portion of FA content was supplied in similar proportion PUFA (~35-40%) or SFA (~30%), with PUFA content reciprocally enriched in n-3 (ie fish oil DHA & EPA ~5:1, NUMEGA Clover Corporation, Altona Australia) or n-6 (ie sunflower oil) class PUFA while the other PUFA class was kept minimal. Thus comparison of fatty acid 'type' replacement was achieved by contrasting SFD (~30% MUFA and ~65% SFA) to PUFA diets (~30% MUFA, ~30% SFA and ~40% PUFA). Similarly, controlled PUFA class replacement was achieved by comparing N3D with N6D. Both diets consisted of similar MUFA / SFA / PUFA proportions as outlined above, and markedly differed in the contrasting n-3 vs n-6 class PUFA component. Diet analysis data are provided to summarize this overall diet contrast strategy in Fig. 1, with detailed fatty acid compositional analyses provided Table 1. These
data demonstrate that a dietary replacement approach to achieve maximal shift in PUFA intake could be achieved.

Animal dietary intervention, tissue harvest & membrane analyses

Male (M) and female (F) Sprague-Dawley rats aged 6 - 8 weeks were randomly allocated to one of three diets N3D, N6D or SFD. Thus 6 treatment groups were defined (N3D/M, N3D/F, N6D/M, N6D/F, SFD/M, SFD/F), with n = 4 - 12 animals each, as specified. Rats were maintained on diets for 4 weeks for myocardial membrane analysis (the minimum 4 week treatment period was based on previously published work showing that this is sufficient period for dietary changes to produce stable membrane phospholipid changes (39)). For ex vivo and in vitro function experiments, male and female rats were maintained on one of the three diets for 8 weeks to allow for membrane composition changes to manifest in downstream outcomes. At the completion of dietary intervention, rats were anaesthetized by 50mg/kg pentobarbitone sodium (i.p.). For ex vivo and in vitro functional experiments, hearts were used immediately (as described in the sections that follow). For heart weight analysis, membrane composition and immunoblot analyses, hearts were excised and weighed for measurement of cardiac weight index (heart weight/ body weight). Left ventricular (LV) tissues were snap frozen in liquid nitrogen until experimental analyses. Total LV ventricular membrane phospholipid fatty acids were extracted from ventricular tissue and analyzed by gas chromatography using a modification of the Folch method as previously described (21).

Cardiomyocyte isolation, Ca\(^{++}\) transient and contractile function measurement

Adult rat ventricular myocytes were enzymatically isolated from diet fed male and female rats as previously described (32). Cell length and width were measured using bright field microscopy (Olympus IX50) at x40 magnification. For each heart one hundred myocytes were randomly selected, and measurements averaged to determine mean myocyte length and
isolated myocytes were loaded with the \( \text{Ca}^{2+} \) fluorescent dye Fura-2AM (1 \( \mu \text{M} \), 20 minutes incubation at 25°C, Sigma Aldrich, USA) and suspended in HEPES-Krebs buffer on an inverted light microscope as previously described (33). \( \text{Ca}^{2+} \) signals as \( (F_{340/380}) \) and shortening kinetics were measured by microfluorimetry and edge detection (video based method of pixel intensity thresholding to track cell end movement at 2ms intervals) as previously reported (IonOptix, Milton, USA) (33). \( \text{Ca}^{2+} \) transient amplitude was determined as the difference between peak systolic and diastolic \( \text{Ca}^{2+} \). Myocyte twitch properties were analyzed for maximum shortening (%S) normalized to diastolic cell length (Ld), maximum rate of shortening (MRS) and lengthening (MRL) normalized to diastolic cell length.

Isolated heart preparation and lactate dehydrogenase analyses

Isolated rat hearts were aerobically perfused and cardiac function was assessed as previously described (6). Briefly, following anaesthesia with pentobarbitone sodium and heparin (50mg/kg and 1000 IU i.p.), hearts were rapidly excised and retrogradely perfused with oxygenated Krebs-Henseleit buffer at 37°C, pH 7.4 (95% \( \text{O}_2 \) – 5% \( \text{CO}_2 \)) at a constant pressure equivalent to 73 mmHg (STH pump controller, AD Instruments, Bella Vista, NSW, Australia). Hearts were perfused aerobically for 30 minutes before 25 minutes of global ischemia (37°C) and 60 minutes of reperfusion. Intrinsic \textit{ex vivo} heart rates were not different. Left ventricular pressure measurements were obtained using a iso-volumetric, intra-ventricular fluid-filled balloon connected to a pressure transducer (ML7844) and data were recorded using a MacLab data acquisition system. The balloon was inflated to produce an end-diastolic pressure of approximately 4 mmHg and the volume was kept constant throughout reperfusion. Data derived include LV developed pressure, maximum rate of contraction (+dP/dt) and relaxation (-dP/dt). At the end of reperfusion, ventricles were
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snap frozen in liquid nitrogen for immunoblot analyses. Coronary effluents were collected throughout the reperfusion protocol.

Lactate dehydrogenase (LDH) content in coronary effluents was measured using a spectrophotometric assay kit (Cytotoxicity Detection kit-PLUS; #04744926001, Roche) as described previously (5). Briefly, coronary effluent samples were assayed against LDH standards (L-lactic dehydrogenase solution, L2625; Sigma-Aldrich). Total LDH released throughout reperfusion (IU) were normalized to heart weight (g) and coronary flow (ml).

Immunoblot analyses

Immunoblot analyses were performed on left ventricular tissues from diet fed perfused and non-perfused rat hearts harvested at the same time and processed in parallel as previously described (33). Tissues were homogenized (10% w/v) in 100mM Tris-HCl buffer with 5mM EGTA and 5mM EDTA (Sigma Aldrich, USA) containing protease and phosphatase inhibitor cocktail (complete protease inhibitor cocktail # 04693159001, Roche; PhosphoSTOP #04906837001, Roche) at 4°C. Tissue homogenate was diluted in 2x sodium dodecyl sulfate (SDS) sample buffer. Protein concentration was measured by a modified Lowry assay to determine equal protein (μg) loading into polyacrylamide gels. Post electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane using a TurboBlot system (Bio-Rad, CA, USA) followed by primary and secondary antibody incubation. Primary antibodies were purchased from Cell Signaling: protein phosphatase 2A - β subunit (PP2A-b) (#2290), Bcl2 associated X protein (Bax) (#2772), Beclin1 (#3738). HRP-conjugated secondary antibody and chemiluminescent reagent (ECL-Plus RPN2133) were purchased from Amersham GE Healthcare. Chemiluminescent signal was imaged and quantified using QuantityOne software (Bio-Rad, CA, USA). To accommodate the large number of samples in 6 treatment groups, multiple gels were required and were run in parallel, each including
protein ladder (Bio-Rad Western C standard, cat # 161-0385) and a common reference standard to ensure that all gel data could be normalized. To verify equal protein loading controls, after imaging membranes were Coomassie stained as per manufacturer’s instructions (Bio-Rad Coomassie Brilliant Blue R-250 Cat # 161-0436), re-imaged in white light and quantified using Bio-Rad QuantityOne software for protein normalization (relative to total protein loading). For blot images, contrast/brightness optimization was applied uniformly to preserve relative densitometric integrity and no non-linear imaging adjustments were made.

**Statistical analyses**

Data are presented as mean ± SEM. Differences between groups were assessed using one-way or two-way ANOVA with repeated measures as appropriate. A diet or sex effect was considered significant when p(diet) or p(sex) or p(diet x sex interaction) < 0.05. Where there was significant factor effect, post-hoc “Fisher’s least significant difference (LSD)” analyses were performed. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL).
RESULTS

Somatic and cardiac growth not influenced by dietary treatment

At the commencement of treatment animals allocated to each diet group exhibited similar
body weights, and growth progression throughout the 8 week treatment period was monitored
and was also similar for all groups. There were no diet-dependent differences in body weight,
heart tissue weight and cardiac weight index (heart weight normalized to body weight)
between diet groups (Fig. 2A-2C). As is usually observed, female body weight was
significantly lower than male body weight, as was heart weight. The cardiac weight index
was significantly higher in females, reflecting the greater proportional difference in body
weight. This relative sex difference in this hypertrophy index is a common observation (6). In
isolated myocytes, there were no differences in length, width and length-to-width ratio
between diet groups or across sexes (Fig. 2D - 2F). Together, these findings confirm that
somatic growth and cardiac trophic growth, in either sex, was not modified by the dietary
interventions and that any dietary functional differences could not be ascribed to alterations
in animal or cardiomyocyte growth characteristics.

Modified dietary PUFA intake and differential myocardial membrane lipid incorporation

The influence of the 3 controlled and contrasting dietary interventions on cardiac tissue
membrane PUFA and SFA incorporation was evaluated (Table 2, Fig. 3). Virtually all lipid
species evaluated exhibited significant differences in relation to diet treatment and sex factor
analysis with most also demonstrating a significant diet x sex interaction. Diet treatments
produced alterations in membrane phospholipid composition which were associated with
remarkable reproducibility between animal samples. In relation to overall dietary factor
analyses, as expected, ventricular membrane n-3 content was higher in N3D compared to
N6D and SFD diet groups, and this n-3 content was comprised almost entirely of DHA. This
n-3 elevation was associated with decreased membrane n-6 PUFA content and a significantly lower n-6:n-3 ratio (Fig. 3A, 3C,3D), demonstrating high potency of N3D in displacing n-6 membrane PUFA. Increased n-6 intake resulted in a higher myocardial n-6 content and n-6:n-3 ratio compared to N3D (Fig. 3A & 3C). Interestingly female arachidonic acid (20:4 n-6) levels were significantly higher in females in both N6D and SFD, with negligible direct dietary delivery (Fig. 3B). The dietary delivery of low n-6 (n-6 ≤ 5% total fatty acid composition) in the SFD produced n-6 membrane content which was similar to the N6D (diet delivery 6-fold higher). This finding evidences membrane retention of n-6 fatty acids even when dietary consumption is relatively low. When SFA ingestion is high, n-6 membrane incorporation levels are not diminished. In contrast with similarly low dietary n-6 intake in an n-3-rich setting, the n-6 membrane levels are reduced by approximately half (Fig. 3B).

In relation to differential male and female myocardial membrane responsiveness to the three diets, important selective shifts were observed. In females, compared with males, increased dietary n-6 (N6D) resulted in more than two-fold increase in membrane n-6:n-3 ratio reflecting both a significant decrease in total n-3 PUFA and an increase in total n-6 PUFA in females (Fig. 3A, 3C, 3D). Also observable was a sex difference in N3D response with more marked n-3 augmentation in N3D females (Fig. 3B and 3D). In terms of the key individual fatty acid(s) that constituted these major membrane PUFA shifts, for n-3 PUFA it was docosahexaenoic acid (DHA), a very long chain n-3 PUFA that predominates the n-3 class PUFA in the myocardial membrane (Table 2); for n-6 PUFA, it was a combination of the medium chain n-6 PUFA linoleic acid (LA) and the long chain n-6 PUFA arachidonic acid (AA).

The overall shifts in membrane lipid proportions is depicted in Fig. 3E and illustrates the phenomenon that myocardial membrane composition is relatively stable in relation fatty acid
types (ie MUFA, PUFA, SFA) whereas within the PUFA component there is considerable
variation in composition in relation to lipid class (ie n-6, n-3). In the SFD group a
dramatically higher SFA intake (67%) compared to 33% in N3D and N6D did not result in
changes in membrane total SFA content (membrane total SFA content was 35%, 35% and
34% for SFD, N3D and N6D male rats (Fig. 1 and Fig. 3E). In females the N6D resulted in
more marked reduction of both n-3 and SFA components than was observed in males (Fig.
3E) and the highest proportion of n-6 was observed.

N6D diet associated with differential effects on basal contractility in males and females
To determine the diet treatment effects on basal cardiomyocyte and cardiac function *in vitro*
and *ex vivo* experiments were performed. Isolated cardiomyocyte shortening and Ca\(^{2+}\)
transients were examined simultaneously (Fig. 4). A significant diet × sex interaction factor
response was observed (in the absence of either single factor diet or sex difference).
Specifically in the N6D group myocyte contractility, maximum shortening and kinetics of
both contraction and relaxation were markedly higher in male compared to female (Fig. 4A-
4D). The maximum rate of shortening, a sensitive index of contractility was more than 60%
elevated in male. In contrast to cell dynamics, no differences in Ca\(^{2+}\) levels could be detected
in myocytes derived from the six treatment groups. Diastolic and Ca\(^{2+}\) transient amplitude
levels were not different (Fig. 4A, 4E, 4F) indicating similar myocyte operational Ca\(^{2+}\) load
levels.

The possibility that increased myofilament Ca\(^{2+}\) sensitivity could explain the increase in
cardiomyocyte contractility in the absence of change in Ca\(^{2+}\) level was considered. At a
single myocyte level, the family of PP2A protein phosphatases have been shown to be
implicated in modifying myofilament sensitivity to modulate contraction in the absence of
Ca\(^{2+}\) transient changes (33). The protein expression of phosphatase PP2A-b was evaluated
and a significant diet × sex interaction factor was also observed, with post-hoc testing 
identifying an N6D specific relative increase in this PP2A in male compared with female 
(Fig. 4G).

In the *ex vivo* heart, basal developed pressure was higher in N6D males compared with 
females (Fig. 5) – consistent with the finding in relation to single myocyte shortening 
responses (Fig. 4C). In this *ex vivo* setting the marked differences in contraction and 
relaxation kinetics were not discernible – in the intact heart the intrinsic cellular dynamics are 
damped and can be less sensitive indices. The discrepancy between the finding in isolated 
myocytes (where shortening kinetics showed sex-difference in N6D) and intact hearts (where 
pressure development rates were not basally different) may also reflect the extent to which 
observations obtained from unloaded and loaded recording settings may not coincide. Taken 
together, these data relating to basal cardiomyocyte and cardiac function (in a setting where 
extrinsic neurohumoral influences are precluded) show that selectively in the N6D myocyte 
and cardiac contractile state is maintained at a higher level in males compared with females, 
with some evidence of underlying phosphatase activity influencing myofilament Ca^{2+} 
sensitivity.

*High n-6 diet abolished intrinsic female ischemia reperfusion acute protection ex vivo*

A role for dietary lipid in influencing acute outcomes after infarct has been identified 
experimentally, when treatments are instituted post infarct (7). To determine the effects of 
longer term diet manipulation on ischemic vulnerability, the recovery of hearts during a 60 
min period following a 25 min global ischemic episode was investigated. Recovery in female 
hearts compared to male hearts was tracked over the 60 min reperfusion period, and 
parameters of developed pressure, maximum rate of contraction and maximum rate of 
relaxation were monitored. In the N6D group, the significant female cardioprotection as
observed in the N3D and SFD groups was abrogated (Fig. 6A-6C). In the N3D and SFD
groups, enhanced post-ischemic resilience in female hearts was characterized as elevation in
developed pressure, and maximum rates of contraction and relaxation over the recovery
period. In N6D females this enhanced resilience was not evident.

To further evaluate the diminished performance of female hearts in the N6D treatment group,
the coronary effluent levels of LDH, a marker of cardiomyocyte necrosis, were measured.
LDH cumulative release over the 60 min reperfusion period was significantly higher in
effluent of N6D female hearts compared to male. The levels were not significantly different
between sexes in the N3D and SFD groups (Fig. 7A-C). Additionally, the occurrence of
arrhythmia in the early reperfusion period was examined for the three diet treatments. This
was calculated as % ectopic beats occurring during the initial 10 minutes of reperfusion. No
differences between diet groups in % ectopy was detected (Fig. 7D). Increased cell death
through necrosis is consistent with the loss of functional recovery observed in the female
N6D group. Pre- and post-ischemic levels of expression of markers known to regulate cell
viability via other survival pathways (apoptosis and autophagy) were examined (Fig. 8). In
non-perfused tissues, not subjected to an ischemic event, a significant diet effect was
observed with lower levels of the expression of Bax (pro-apoptotic) and Beclin-1 (autophagy
initiator) in N3D compared with both N6D and SFD. At the 60 min acute post-ischemia
timepoint no dietary differences in the relative level of expression of these markers was
evident. No sex differences in either pre- or post-ischemic expression levels were detected.
DISCUSSION

In this investigation the sex-specific cardiac functional effects of three fully specified diets of different lipid composition were evaluated. Diets were designed to achieve contrast of lipid type by manipulating fatty acid replacement (saturated / monounsaturated / polyunsaturated) fatty acid components. Within the polyunsaturated diet type, the effect of omega fatty acid class substitution was also determined (i.e. n-6 vs n-3). When fed identical diets cardiac membrane phospholipid fatty acid composition profiles were different in males and females.

In females the myocardial membrane n-6:n-3 PUFA ratio was two-fold higher than male (Fig. 3) in the n-6 diet replacement group (N6D). In diets specified to be high in omega-3 PUFA or in saturated fat, this sex difference was not apparent. Ex vivo and in vitro measures of myocardial function and cardiomyocyte performance exhibited consistent sex differences within the omega-6 diet group which were not observed in the omega-3 or saturated fat diet groups. Female omega-6 fed rat hearts and myocytes demonstrated reduced contractile performance under basal conditions relative to male, and after ischemic insult female resilience in reperfusion recovery was impaired. This response was associated with evidence of increased ischemic necrotic damage/cell loss evaluated by coronary LDH release during reperfusion. Cardiac and myocyte functional parameters were not different between omega-3 and saturated fat dietary groups and within these groups there were no discernible sex differences. These findings show that replacement of dietary saturated fat with n-6 PUFA (but not n-3 PUFA) undermines female cardioprotection in ischemia reperfusion.

Cardiac membrane lipid composition profiles in males and females
The confirmation of equivalent somatic and cardiac growth trajectories across diet groups for each sex validated the stringent design approach to generate nutritionally well matched diets distictively differentiated by selective fatty acid replacement. The 'replacement'
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characteristics of the diets were well defined in relation to FA type and PUFA class – and interpretation is thus free of confounding 'supplement' effects. As has been previously reported in rodent and human, this study confirmed that with the N3D, total membrane n-3 PUFA was elevated and n-6:n-3 ratio decreased (30, 34). A higher level of cardiac n-3 membrane incorporation with n-3 PUFA feeding in female rodents has also been reported with elevated α-linolenic acid (18:3 n-3) intake and attributed to increased hepatic expression of desaturase and elongase enzymes involved in long chain FA synthesis providing increased plasma FA availability for cardiac uptake (23). The cardiac membrane fatty acid content compared across the groups affirms the understanding that proportions of total MUFA, SFA and PUFA vary only slightly, regardless of the major differences in the dietary lipids among the three groups (Fig. 1 and Fig. 3E), indicating a strict regulation of membrane lipid ‘types’ (ie SFA vs MUFA vs PUFA) incorporated to maintain biophysical properties. There was a dominant membrane incorporation of n-6 PUFA (ranging from 43-52%) even in the context of a large shift in n-6 dietary source from <4.5% (in SFD) to almost 40% (N6D). In contrast, with N3D treatment, membrane n-6 PUFA was markedly reduced to 23-28% indicating responsiveness to displacement of n-6 PUFA by n-3 PUFA. The present findings show that myocardial arachidonic acid (20:4 n-6) levels were significantly elevated in N6D and SFD females compared to males. Arachidonic acid levels in N6D and SFD groups were greater in all males and females respective to N3D group counterparts. Although higher rates of desaturase activity might account for the greater proportion of arachidonic acid in females, notably, there was no significant increase in arachidonic acid (20:4 n-6) membrane levels in N3D females compared to males.

The key sex-specific membrane composition difference was a significant 2-fold disparity in membrane n-6:n-3 ratio demonstrated in the N6D. This increase in ratio was the outcome primarily of lower total n-3 and higher total n-6 (particularly arachidonic acid) in female
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393   N6D, relative to male. With n-3 diet supply low and n-6 supply high, membrane n-6
394   incorporation was promoted in both males and females. Furthermore, with high dietary n-3
395   PUFA and low n-6 PUFA, although there was marked membrane uptake of n-3 PUFA in
396   males and females, this was greater in females, at the expense of arachidonic acid. It is also
397   feasible that a sex difference in arachadonic acid metabolism (involving phospholipase A2
398   and/or cyclo-oxygenase activity could be involved in determining membrane arachidonic
399   acid levels. It is important to note that dietary lipid abundance and the dietary ratio of n-6:n-3
400   PUFA per se may have no influence on long chain n-3 PUFA myocardial membrane
401   incorporation, as has been shown previously even with very low levels of dietary fish oil (43).
402   Whether females have different rates of desaturase and elongase activities in response to
403   competition and shifts between dietary PUFA class is unclear. This conclusion is beyond the
404   scope of this study, and awaits future investigation. However, it appears that cardiac
405   membranes of females have greater requirement for long chain PUFA, incorporating more
406   arachidonic acid in N6D and more n-3 PUFA (mainly DHA) in N3D, according to abundance
407   of the longest PUFAs. Compared to males, in N6D females an n-3 membrane ‘deficiency’
408   effect is significantly more pronounced. This crucial sex-specific membrane composition
409   difference was linked to functional difference at heart, cardiomyocyte and subcellular protein
410   level.

411   **Female omega-6 fed hearts and myocytes exhibit diminished contractile performance**
412   A significant basal performance difference was observed between hearts and cardiomyocytes
413   of N6D males and females. Interestingly, this differential can be largely attributed to higher
414   level myocyte kinetic and intact heart contractility performance in males not matched by
415   females. In female cardiomyocytes, we and others have previously reported lower levels of
416   cell shortening than in males, although this is not a universal finding (4, 40). It seems
417   probable that dietary context may have an important role in determining when sexual

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dimorphism in single cardiomyocyte contractile function is evident. Some mechanistic insight is derived from the finding that the N6D male and female myocyte Ca\textsuperscript{2+} transients are not different. The higher level of expression of the protein phosphatase PP2A-b in the N6D males is consistent with augmented contractile myofilament Ca\textsuperscript{2+} responsiveness. Increased abundance of PP2A family proteins (by genetic manipulation and diabetic dietary induction) has been previously linked with increased contractile response in the absence of change in the Ca\textsuperscript{2+} transient (22, 33). There is also evidence of estrogen regulation of PP2A family genes in the heart, consistent with observations that in a relatively estrogenic state PP2A activity is lower (4). However, PP2A has a multitude of targets including Ca\textsuperscript{2+} handling proteins, and a contrasting PP2A effect was also apparent in females fed SFD even though no differential shortening or transient actions were evident. An interaction between dietary PUFA and estrogen status provides for considerable nuance in regulating cardiac function. A mechanism involving elevated n-6 dietary intake and enhanced PP2A family protein activation requires elucidation, including direct investigation of myofilament protein phosphorylation status.

It is notable that evidence of detrimental SFD effect on cardiac function was absent – as was evidence of beneficial effect of N3D. This finding coincides with emergence of recent questions regarding the efficacy of SFA dietary exclusion (28, 36). Experimentally, this matter requires further examination using comparative ex vivo models - it is possible that working heart and Langendorff models offer different functional perspectives. The literature arising from dietary studies using isolated heart models, has not yet generated consistent findings (1, 18, 41).

**Dietary omega-6 impairs functional recovery after ischemia in females**

After ischemic insult, hearts of N3D and SFD females exhibited markedly improved acute recovery at the 60 min reperfusion timepoint compared with diet matched males – but hearts
of N6D females did not. The differential capacity for post-ischemic acute recovery in N6D
males and females may partly derive from the same mechanistic origin involved in
differential basal contractile state – estrogen influences may operate to suppress contractility
recovery at the myofilament level in an n-6 enriched environment. In non-dietary studies, we
and others have previously reported that female hearts show greater resilience in recovery
immediately after ischemia compared with males (6, 15, 37). An analysis of the reliance of
this differential response on dietary status has not been previously undertaken. An earlier
study investigating female mice only, has found no difference between n-6 and n-3 enriched
diet on the post-ischemic ex vivo cardiac recovery in intact and in ovariectomized mice (21).
In that study, a direct sex comparison was not made. The dietary intervention undertaken in
the murine study did not involve a 3-way comprehensive diet contrast as utilized in the
present study. Species may also be a relevant factor in identifying diet-dependent ischemic
resilience – more substantial shifts in rat and human membrane lipid composition can be
achieved by dietary intervention than is possible in the mouse. Rat and human sex-specific
tissue lipid profiles have been shown to be relatively similar, while mouse n-3 membrane
PUFA n-3 incorporation is higher with diets not enriched for n-3 (8, 10, 23). Thus, these
present findings of N6D induced sex-difference in acute ischemic resilience in the rat may
provide a finding of most direct human relevance.

Differential diet effects on cardiomyocyte viability and viability signaling

The impaired resilience of female N6D hearts in recovery after ischemia occurred in parallel
with a markedly higher coronary effluent release of LDH in female compared with male diet
matched hearts, signifying higher level of necrotic cell injury/death during the ischemic
period – even though the insult period was relatively mild. Cell death by necrosis is a
common feature of the ischemic response (2). This finding indicates greater vulnerability of
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females exposed to N6D treatment than males. The less robust systolic recovery in
reperfusion likely reflects this increased extent of necrotic damage. The lower N6D female
recovery after ischemia cannot be attributed to differential arrhythmogenic activity in
reperfusion, as the level of ectopy was shown to be similar for all groups. A differential sex
contribution of formation of n-6-derived highly reactive peroxidation products (ie 4-hydroxy-
2-nonane, 4-HNE) may also be relevant. Whilst the n-3 oxidation product (4-hydroxy-
hexenal, 4-HHE) is reported to exert protective effect, there is evidence that necrotic cell
death responses are activated by 4-HNE – and this may be amplified in the enriched n-6
environment during conditions of oxidative stress (2, 38). It is possible that different dietary
regimes are associated with different levels of exogenous intake of peroxidation and
oxidation agents – this was not determined in the present study. The present study does not
provide direct evidence to address this question, and a thorough assessment of
necrotic/oxidative damage pathways associated with N6D intervention is now warranted. It
should be noted that previous investigation of the effects of AA on cell death markers have
been shown not to be detrimental (27). AA is certainly an important nutritional FA, which
when provided via diet by enrichment may have different effects than when supplied in the
form of a 'replacement' component. It should also be noted that prior experimental studies
have not involved sex comparisons (17).

Examination of the levels of molecular markers associated with induction of other cell death
signaling pathways revealed clear dietary, but not sex-specific differences. Bax is a well
characterized pro-apoptotic protein, levels of which are indicative of programmed cell death
induction. Under basal conditions (ie in tissues not subjected to ischemia) Bax levels were
significantly lower in N3D relative to both N6D and SFD. Autophagy is a process of
phagocytic/lysosomal degradation elevated in stress, initiated by beclin-1, and linked to
programmed cell death when upregulated in a sustained manner (12). Beclin1 protein

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expression was also found to be significantly lower in the N3D groups compared to N6D and SFD. Both the Bax and Beclin1 findings are consistent with an n-3 mediated suppression of constitutive programmed cell death signalling – a situation which may limit progressive myocyte loss, in the absence of a specific injury event. Post-ischemia the induction of signalling through both pathways was very substantially elevated, and in this setting subtle differences in expression levels were not discernable. Thus, although evidence of n-3 benefit in acute functional recovery after ischemia was absent, the possibility that longer term cardiomyocyte viability may be promoted by n-3 enrichment is apparent. This proposition requires direct investigation, potentially using histological methodologies which may be more definitive. Recent findings also suggest that N6D treatment may play a myocardial pro-inflammatory role post-infarct, and this is also an area of valid future investigation (29).

In conclusion, in this study we have developed diets to specifically manipulate omega-3 and omega-6 PUFA classes within a defined PUFA component, in order to contrast the effects of these treatments with a matched diet of near equivalent saturated fatty acid composition. A marked elevation in the omega-6:omega-3 PUFA ratio of membrane lipid incorporation in females was identified with omega-6 PUFA enriched diets. This sex difference was only evident when omega-6 PUFA were provided in excess, and not with diets specified to be high in either omega-3 PUFA or saturated fat which were concomitantly low in n-6 PUFA. The present findings identify a requirement in females for a greater proportion of long chain PUFA within the total PUFA membrane compartment, such that more arachidonic acid is incorporated from omega-6 PUFA-rich diet (N6D) or more omega-3 PUFA (mainly DHA) is incorporated from an n-3 PUFA-rich diet (N3D). Functional studies demonstrated significant basal performance deficit in female cardiomyocytes and isolated hearts of animals fed omega-6 enriched diets compared to male comparators, and impaired functional resilience during reperfusion after ischemia. Our findings provide evidence at the myocardial and
cellular level that dietary saturated fatty acid intake replacement with an omega-6 (but not omega-3) enriched diet has selective detrimental cardiac functional effect in females. The observation that females can accumulate greater myocardial membrane n-3 PUFA when available, may underlie the enhanced capacity of females to tolerate acute ischemia-reperfusion injury in dietary settings where omega-3 dietary intake is not limiting. Collectively these findings suggest that high omega-6 dietary lipid intake in females may adversely impact cardioprotection status.
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ACKNOWLEDGEMENTS

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REFERENCES


FIGURE CAPTIONS

Fig. 1. Fabricated rodent diet fatty acid composition depicting proportion of total lipid by type and by class.

A: Omega-3 diet (N3D);
B: Omega-6 diet (N6D);
C: Saturated fat diet (SFD).

Data presented are % total fat.

Fig. 2. Somatic, cardiac and cardiomyocyte size measures from male and female rats fed n-3- (N3D), n-6- (N6D) or saturated fat (SFD)-enriched diet at completion of 8 week dietary treatment.

A-C: Sex differences, but no diet differences observed in body weight, heart weight, cardiac weight index.
D-F: No effect of diet or sex on myocyte size was detected as determined by mean myocyte length, width and length:width ratio.

Data presented: n ≥ 7 / group, data presented as median + Tukey inner fences, two-way ANOVA, *p(sex)<0.05 (A-C); n = 200-300 myocytes / group, data presented as median + Tukey inner fences, two-way ANOVA, p=ns. (D-F) Cardiac weight index = heart weight / body weight (mg/g).
Fig 3. Myocardial membrane fatty acid (FA) composition following dietary intervention.

A: Increased omega-6:omega-3 ratio in female rats compared to males fed N6D.
B: Highest levels of membrane AA in N6D/F compared to N6D/M.
C: Lowest level of membrane total omega-6 PUFA in N3D.
D: Total omega-3 content (comprised almost entirely of DHA).
E: Representation of total membrane lipid MUFA, PUFA & SFA types, also identifying PUFA omega-3 and omega-6 classes.

Data presented: percentage of total membrane phospholipid fatty acids (mean ± SEM, n ≥ 4 hearts/group), two-way ANOVA *p(diet)<0.05, *p(diet x sex)<0.05, post-hoc LSD. SEM bars not visible where variance very low. AA, arachidonic acid.

Fig 4. Myocyte twitch, Ca²⁺ transient and PP2A expression.

A: Representative records of myocyte twitch and Ca²⁺ transients.
B-D: Myocyte twitch amplitude (% shortening) and the maximum rate of shortening and lengthening were selectively higher in N6D/M cardiomyocytes compared to diet-matched female (N6D/F).
E-F: No diet or sex difference was detected in diastolic Ca²⁺ or Ca²⁺ amplitude.
G: Protein expression of PP2A-b was higher in N6D/M vs N6D/F.
H: Coomassie stained PP2A-b membrane showing equal protein loading.
I: Representative PP2A-b blot image.

All samples were derived at the same time and were processed in parallel. Bands have been rearranged to match graph layout. Common calibrator samples incorporated in all gels to ensure inter-gel quantification integrity. Data presented: mean ± SEM (n ≥ 7/group), two-way ANOVA *p(diet x sex)<0.05, p(post-hoc LSD)<0.05. Lo, resting myocyte length; PP2A-b, protein phosphatase 2A - B subunit.
Fig. 5. Basal ex vivo basal cardiac performance.
A: Increased n-6 PUFA intake associated with higher developed pressure in males but not females.
B-C: No diet or sex differences detected in maximum rate of contraction and relaxation.
Data presented: mean ± SEM (n ≥ 7 / group), two-way ANOVA (diet x sex interaction).
*p<0.05 post hoc LSD (sex).

Fig. 6. Ex vivo contractile function recovery throughout 60 min of reperfusion.
A-C: Female hearts from N3D and SFD exhibited significantly higher functional recovery compared to diet-matched males. This female-specific enhanced recovery in reperfusion was absent in N6D/F.
Data presented: mean ± SEM (n = 8 / group), one-way ANOVA with repeated measures.
*p<0.05 post hoc LSD (sex). AP, aerobic perfusion

Fig. 7. Necrosis marker release during 60 min reperfusion.
A-C: Necrosis marker release during 60 min reperfusion. A high level of coronary lactate dehydrogenase (LDH) release, indicative of necrotic myocyte rupture detected in N6D/F female hearts but not in N3D and SFD. Data presented: LDH activity (U) normalised to coronary flow (L) and heart weight (g) (mean ± SEM), n = 8 / group av, one-way ANOVA with repeated measure, *p<0.05 post hoc LSD (sex).
D: % Ectopy measured during initial 10min reperfusion. No dietary or sex differences in arrhythmogenic responses. Data presented: mean ± SEM (n ≥ 7 / group), two-way ANOVA, p=ns.
Fig. 8. Cell viability signalling markers in non-perfused and in ischemia-reperfused myocardium.

A: Equal protein loaded immunoblots showing non-perfused (pre-ischemia equivalent) and post ischemia-reperfusion (post i/r) myocardial Bax and Beclin1; Coomassie-stained membrane in lower panel.

B, D: Non-perfused myocardial protein expression of Bax and Beclin1.

C, E: Post i/r myocardial protein expression of Bax and Beclin1.

All samples were derived at the same time and were processed in parallel. Bands have been rearranged to match graph layout. Common calibrator samples incorporated in all gels to ensure inter-gel quantification integrity. Data presented: calibrator sample normalized, background-subtracted chemiluminescent signal (n = 7/group), two-way ANOVA (diet), $\#p<0.05$ post hoc LSD (diet).
TABLE 1

Fabricated rodent diet fatty acid composition analysis by gas chromatography.

Data presented as % total diet lipid (n = 3 samples / diet). Total SFA (total saturated fatty acid); Total MUFA (total monounsaturated fatty acid); Total PUFA (total polyunsaturated fatty acid); Total omega-6 PUFA (total omega-6 polyunsaturated fatty acid); Total omega-3 PUFA (total omega-3 polyunsaturated fatty acid). Major (but not all) FA species tabulated.

<table>
<thead>
<tr>
<th>Fatty acid &amp; common name</th>
<th>n-3 enriched diet (N3D)</th>
<th>n-6 enriched diet (N6D)</th>
<th>SFA enriched diet (SFD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behenic</td>
<td>3.1</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Stearic</td>
<td>18:0</td>
<td>5.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Palmitic</td>
<td>16:0</td>
<td>19.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Myristic</td>
<td>14:0</td>
<td>2.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Lauric</td>
<td>12:0</td>
<td>0.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Total SFA</td>
<td>32.2</td>
<td>32.5</td>
<td>65.2</td>
</tr>
<tr>
<td>Gadoleic</td>
<td>20:1 n-9</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Oleic</td>
<td>18:1 n-9</td>
<td>27.9</td>
<td>27.4</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>16:1 n-7</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>31.5</td>
<td>27.9</td>
<td>28.9</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>20:4 n-6</td>
<td>1.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Linoleic</td>
<td>18:2 n-6</td>
<td>3.7</td>
<td>38.8</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>5.2</td>
<td>38.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>22:6 n-3</td>
<td>24.7</td>
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<tr>
<td>Eicosapentaenoic</td>
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<td>α-linolenic</td>
<td>18:3 n-3</td>
<td>0.5</td>
<td>0.4</td>
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<td>Total n-3 PUFA</td>
<td>29.8</td>
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<td>0.3</td>
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<tr>
<td>Total PUFA</td>
<td>34.7</td>
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<td>4.5</td>
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<tr>
<td>Total n-6 : total n-3</td>
<td>0.2</td>
<td>97.0</td>
<td>15.8</td>
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<tr>
<td>Total SFA : total PUFA</td>
<td>0.9</td>
<td>0.8</td>
<td>14.4</td>
</tr>
</tbody>
</table>
TABLE 2.

Membrane lipid composition analysis. Data presented are percentage of total membrane phospholipid fatty acids (mean ± SEM, n ≥ 4 hearts / group) except for n-6:n-3 (a ratio). Fatty acid terminology - CX:Y n-Z represents the length of the carbon chain (X), the number of carbon double bonds (Y), and the position of first carbon double bond relative to methyl-terminal end (n-Z). Sum of all tabulated fatty acids does not equate 100% because fatty acids in very low abundance are not listed and comprise ≤3% total membrane fatty acid. Abbreviations: LA, linoleic acid; ALA, alpha linolenic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexanoic acid; ND, not detected. # denotes significant diet effect; * denotes significant sex effect; † denotes significant diet and sex interaction.

<table>
<thead>
<tr>
<th>Fatty acids &amp; common name</th>
<th>n-3-enriched diet (N3D)</th>
<th>n-6-enriched diet (N6O)</th>
<th>SFA-enriched diet (SF0)</th>
<th>ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>11.33 ± 0.14</td>
<td>11.03 ± 0.26</td>
<td>9.94 ± 0.19</td>
<td>5.59 ± 0.36</td>
</tr>
<tr>
<td>C16:1 n-7 Palmitoleic</td>
<td>0.67 ± 0.02</td>
<td>ND</td>
<td>0.27 ± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>C17:0 Margaric</td>
<td>0.64 ± 0.02</td>
<td>0.53 ± 0.04</td>
<td>0.18 ± 0.01</td>
<td>0.77 ± 0.15</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>22.13 ± 0.25</td>
<td>22.33 ± 0.55</td>
<td>22.97 ± 0.19</td>
<td>21.47 ± 0.54</td>
</tr>
<tr>
<td>C18:1 n-7 Vaccenic</td>
<td>3.88 ± 0.08</td>
<td>3.92 ± 0.14</td>
<td>3.63 ± 0.10</td>
<td>3.07 ± 0.39</td>
</tr>
<tr>
<td>C18:1 n-9 Oleic</td>
<td>5.33 ± 0.13</td>
<td>5.19 ± 0.39</td>
<td>4.28 ± 0.08</td>
<td>5.87 ± 0.13</td>
</tr>
<tr>
<td>C18:3 n-6 Linoleic (LA)</td>
<td>10.12 ± 0.28</td>
<td>5.19 ± 0.24</td>
<td>20.68 ± 0.44</td>
<td>17.70 ± 0.95</td>
</tr>
<tr>
<td>C18:3 n-3 Alpha-linolenic (ALA)</td>
<td>0.06 ± 0.00</td>
<td>0.22 ± 0.02</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>C18:3 n-6 Gamma-linolenic (GLA)</td>
<td>0.04 ± 0.00</td>
<td>0.11 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>C20:1 n-6 Dihomo-gamma-linolenic</td>
<td>0.34 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.32 ± 0.05</td>
</tr>
<tr>
<td>C20:4 n-6 Arachidonic (AA)</td>
<td>16.44 ± 0.34</td>
<td>16.38 ± 0.59</td>
<td>23.98 ± 0.56</td>
<td>29.05 ± 0.46</td>
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<tr>
<td>C20:5 n-3 Eicosapentaenoic (EPA)</td>
<td>1.30 ± 0.06</td>
<td>2.44 ± 0.22</td>
<td>ND</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>C22:5 n-3 Docosapentaenoic n-3</td>
<td>1.14 ± 0.02</td>
<td>1.77 ± 0.34</td>
<td>1.11 ± 0.07</td>
<td>0.31 ± 0.26</td>
</tr>
<tr>
<td>C22:5 n-6 Docosapentaenoic n-6</td>
<td>0.81 ± 0.01</td>
<td>0.78 ± 0.02</td>
<td>3.17 ± 0.15</td>
<td>3.46 ± 0.14</td>
</tr>
<tr>
<td>C22:6 n-3 Docosahexanoic (DHA)</td>
<td>24.43 ± 0.39</td>
<td>17.75 ± 0.46</td>
<td>7.28 ± 0.27</td>
<td>4.03 ± 0.32</td>
</tr>
<tr>
<td>n-6:n-3</td>
<td>1.04 ± 0.03</td>
<td>0.72 ± 0.04</td>
<td>3.50 ± 0.27</td>
<td>11.81 ± 0.27</td>
</tr>
</tbody>
</table>
Fig. 1. Fabricated rodent diet fatty acid composition depicting proportion of total lipid by type and by class.
A: Omega-3 diet (N3D);
B: Omega-6 diet (N6D);
C: Saturated fat diet (SFD).
Data presented are % total fat.
Fig. 2. Somatic, cardiac and cardiomyocyte size measures from male and female rats fed n-3- (N3D), n-6- (N6D) or saturated fat (SFD)-enriched diet at completion of 8 week dietary treatment. A-C: Sex differences, but no diet differences observed in body weight, heart weight, cardiac weight index.

D-F: No effect of diet or sex on myocyte size was detected as determined by mean myocyte length, width and length-width ratio.

Data presented: n = 7 / group, data presented as median + Tukey inner fences, two-way ANOVA.

*p(sex)<0.05 (A-C); n = 200-300 myocytes / group, data presented as median + Tukey inner fences, two-way ANOVA, p<ns. (D-F) Cardiac weight index = heart weight / body weight (mg/g).
Fig. 3. Myocardial membrane fatty acid (FA) composition following dietary intervention.
A: Increased omega-6:omega-3 ratio in female rats compared to males fed N6D.
B: Highest levels of membrane AA in N6D/F compared to N6D/M.
C: Lowest level of membrane total omega-6 PUFA in N3D.
D: Total omega-3 content (comprised almost entirely of DHA).
E: Representation of total membrane lipid MUFA, PUFA & SFA types, also identifying PUFA omega-3 and omega-6 classes.

Data presented: percentage of total membrane phospholipid fatty acids (mean ± SEM, n ≥ 4 hearts/group), two-way ANOVA *(p( diet)<0.05, *p (diet x sex)<0.05, post-hoc LSD. SEM bars not visible where variance very low. AA, arachidonic acid.
Fig. 4. Myocyte twitch, Ca\textsuperscript{2+} transient and PP2A expression.
A: Representative records of myocyte twitch and Ca\textsuperscript{2+} transients. B-D: Myocyte twitch amplitude (% shortening) and the maximum rate of shortening and lengthening were selectively higher in N60/M cardiomyocytes compared to diet-matched female (N60/F). E-F: No diet or sex difference was detected in diastolic Ca\textsuperscript{2+} or Ca\textsuperscript{2+} amplitude. G: Protein expression of PP2A-b was higher in N60/M vs N60/F. H: Coomassie stained PP2A-b membrane showing equal protein loading. I: Representative PP2A-b blot image. All samples were derived at the same time and were processed in parallel. Bands have been rearranged to match graph layout. Common calibrator samples incorporated in all gels to ensure inter-gel quantification integrity. Data presented: mean ± SEM (n ≥ 7/group), two-way ANOVA *p(diet x sex)<0.05, post-hoc LSD)<0.05. Lo, resting myocyte length; PP2A-b, protein phosphatase 2A - B subunit.
FIGURE 5.

A. Developed pressure

B. Max rate of contraction

C. Max rate of relaxation

Fig. 5. Basal ex vivo basal cardiac performance.

A: Increased n-6 PUFA intake associated with higher developed pressure in males but not females.

B–C: No diet or sex differences detected in maximum rate of contraction and relaxation.

Data presented: mean ± SEM (n = 7/group), two-way ANOVA (diet x sex interaction), *p<0.05 post hoc LSD (sex).
Fig. 6. Ex vivo contractile function recovery throughout 60 min of reperfusion.
A-C: Female hearts from N3D and SFD exhibited significantly higher functional recovery compared to diet-matched males. This female-specific enhanced recovery in reperfusion was absent in N3D/F.
Data presented: mean ± SEM (n = 8/group), one-way ANOVA with repeated measures, *p<0.05 post hoc LSD (sex). AP, aortic perfusion.
Fig 7: Reperfusion injury and arrhythmia occurrence.

A-C: Necrosis marker release during 60 min reperfusion. A high level of coronary lactate dehydrogenase (LDH) release, indicative of necrotic myocyte rupture detected in N6D-female hearts but not in N3D and SFD. Data presented: LDH activity (U/L) normalised to coronary flow (L) and heart weight (g) (mean ± SEM), n = 8 / group av, one-way ANOVA with repeated measure, *p<0.05 post hoc LSD (two).

D: % Ectopy measured during initial 10 min reperfusion. No dietary or sex differences in arrhythmogenic responses. Data presented: mean ± SEM (n ≥ 7 / group), two-way ANOVA, p<0.05.
Fig. 8. Cell viability signalling markers in non-perfused and in ischemia-reperfusion myocardium. 
A: Equal protein loaded immunoblots showing non-perfused (pre-ischemia equivalent) and post ischemia-reperfusion (post i/r) myocardial Bax and Beclin1. Coomassie-stained membrane in lower panel. 
B, D: Non-perfused myocardial protein expression of Bax and Beclin1. 
C, E: Post i/r myocardial protein expression of Bax and Beclin1. 
All samples were derived at the same time and were processed in parallel. Bands have been rearranged to match graph layout. Common calibrator samples incorporated in all gels to ensure inter-gel quantification integrity. Data presented: calibrator sample normalized, background-subtracted chemiluminescent signal (n = 7/ group), two-way ANOVA (diet), *p<0.05 post hoc LSD (diet).
CHAPTER 6
GENERAL DISCUSSION

The overall goal of this Thesis (submitted with 3 integrated publications) was to determine whether dietary polyunsaturated fatty acid (PUFA) confers cardiac benefit (when substituted for SFA), and if omega-3 compared to omega-6 augmentation produces distinctive effect. In light of the inconsistent literature regarding the cardiac benefit / detriment of dietary omega-3 PUFA, this study has been designed to address the key limitations in the experimental and clinical literature. Firstly, a pre-surgical n-3 PUFA supplementation study was performed to investigate the implications of a common flaw in dietary interventional study design - the over-simplistic comparison of a ‘n-3 supplemented’ vs ‘placebo’ groups without treatment compliance verification. Secondly, a set of stringently designed experimental diets were fabricated to compare three distinctive fatty acid replacement regimes in male and female rats. This approach enabled interpretation beyond the common fatty acid ‘enrichment’ effect, and also allowed evaluation of the effects of shifts in specific dietary fatty acid intake, including selective replacement of fatty acid type and class. Preceding these two studies a methodological investigation was undertaken to validate usage of tissues subjected to long-term archiving for experimental analyses of dietary FA intervention.

Findings of this study will aid to demystify current understanding about experimental fatty acid efficacy on cardiac function, and have important relevance to women, cardiac risk and dietary management. In this discussion, a brief overview of the main findings of the papers presented for examination is provided and an additional commentary in relation to result interpretation and clinical implication is offered.
6.1 **OMEGA-3 SUPPLEMENTATION: A CLINICAL STUDY**

Since the early population studies linking high consumption of marine n-3 PUFA with lowered incidence of coronary disease and mortality, epidemiological and observational studies have reported increased fish intake to be of cardiac benefit. However, more recent randomized control trials (RCTs) have not reconciled with these findings (see Chapter 2 Literature Review and Chapter 4 Publication #2 for details). With RCTs, interpretation is potentially confounded by background n-3 PUFA intake.

The goal of the first study was to test the effectiveness of clinical treatment randomization in producing well-contrasted n-3 treatment groups. Patients were randomized to receive 3g / day of fish oil (FO) by capsule or placebo oil (PO). Patient end-treatment tissue molecular measures and post-operative atrial fibrillation (AF) was evaluated. Substantial overlap in the level of membrane EPA and DHA in PO and FO groups was observed. Whilst significant correlations between cardiac tissue expression levels of molecular markers involved in myocardial viability and metabolic signaling in ischemia stress and n-3 PUFA content were observed in the pooled patient cohort, no PO or FO group treatment effects could be discerned. Stratification of patient data on the basis of myocardial membrane EPA+DHA level (using a threshold value of 8% total membrane lipid) was demonstrated to produce an effective and significant dichotomous separation of patients (based on established omega-3 index). Stratification slightly enhanced the AF group differential compared to randomization group values, but significant AF benefit of omega-3 status was not detectable in either analysis setting.

The key finding of this clinical study was that in relation to omega-3 efficacy, the same cohort may present different findings when evaluated by treatment randomization and by stratification to actual myocardial membrane fatty acid. The study demonstrated that even in a small patient cohort, evaluation of omega-3 supplementation efficacy by stratification method based on erythrocyte n-3 content has the capacity to generate more decisive findings in relation to cardiac endpoint analysis than can be achieved through reliance on randomization group analysis.
Based on the findings in this study, the following recommendations in relation to future clinical trials can be made:

To determine PUFA treatment efficacy, it may be more informative to perform post-hoc regression analysis, and compare tissue PUFA content to study a designated endpoint. In addition, studies should maximize differences in n-3 tissue levels between control and intervention group, and apply the following exclusion criteria:

1. Participants who regularly consume more than 1 fish meal / week;
2. Participants who are self-administering any type of n-3 or n-6 supplement (e.g. fish oil, cod liver oil, krill oil) and are unwilling to discontinue their use;
3. Participants who cannot confirm that they will not use n-3 or n-6 supplements during the course of study;
4. Exclude participants whose erythrocyte membrane n-3 content returns a value greater than 8%

6.2 **Omega 3 Replacement: An Experimental Study**

Investigation of cardiac-specific effect of dietary fatty acids in animal models has generated mixed results (see Chapter 2 Literature Review and Chapter 5 Publication #3 for details). The reasons for controversies include the use of undefined ‘standard chow’ diets as reference; and the caloric and oxidative effects of using oil additives to shift diet lipid profiles, which can confound study findings. In addition to the controversial cardiac effect of dietary FA, there is an emerging clinical literature suggestive of sex-related differences in response to lipid dietary intervention. To date the sex-selective effects of dietary FA has not been independently investigated.

For this study, three rodent diets were developed that involved specific manipulation of n-3 and n-6 PUFA class within a defined PUFA component, to contrast the effects of these treatment with a matched diet of near equivalent saturated fatty acid
composition. The goals of this study were two-fold. First, to determine the effect of dietary fatty acid type and/or class replacement in male rats (as reference). Second, to determine the sex-selective effect of dietary fatty acid replacement. Male and female rats were fed one of the three diets specified to maximise contrast between FA type (i.e. SFA vs PUFA diets) and between FA class (n-3 vs n-6) for 8 weeks. Investigation of performance of heart and cardiomyocytes derived from male and female rats fed one of the three diets was undertaken.

In rats that were fed the same diet, significant sex differences in myocardial membrane lipid composition were found. A sex-specific dietary fatty acid response was observed in omega-6 diet group (N6D), where the myocardial membrane n-6:n-3 ratio was two-fold higher in female compared to male. This sex difference in n-6:n-3 ratio was not apparent in omega-3 diet group (N3D), or saturated fat diet group (SFD). Further evaluation of ex vivo and in vitro myocardial function and myocyte performance showed similar omega-6 specific sex differences that were not observed in the omega-3 or saturated fat groups. Omega-6 fed female rat hearts and myocytes showed lower contractile function under basal conditions relative to diet-matched males, and following ischemic insult, intrinsic female resilience in reperfusion recovery was significantly diminished. This was associated with an increased ischemic necrotic cell damage/loss, as determined by coronary LDH release during reperfusion. In omega-3 and saturated fat groups, no discernible sex differences were observed in cardiac and myocyte functional parameters measured.

Collectively these findings demonstrate that replacement of dietary saturated fat with n-6 PUFA, but not n-3 PUFA, selectively erodes female cardioprotection in ischemia reperfusion. Whilst no experimental studies have investigated in the sex-selective actions of dietary n-3 and n-6 PUFA replacement, Ramsden et al recently reported clinical evidence of potential detrimental effect of high n-6 intake in women (and not men) (Ramsden et al. 2016). The authors analyzed newly recovered information related to participant sex and age from the Minnesota Coronary Experiment (MCE) – one of the most rigorously executed dietary trial designed to investigate effect of replacing dietary saturated fat with n-6 linoleic acid for prevention of cardiovascular
events and death (Frantz et al. 1989). The MCE is the only RCT to complete post-mortem assessment of cardiovascular endpoints, and the only trial to test the clinical effects of augmentation of n-6 linoleic acid in large pre-specified subgroups of women (46% women, n = 1083) and older adults. Ramsden et al found that in women who received the saturated fat replacement regime (i.e. high n-6 PUFA linoleic acid in replace of saturated fat) had higher mortality over 4-years follow-up, compared to women who did not consume the saturated fat replacement diet. Whilst no statistical analysis was performed due to the absence of original data, the newly recovered finding is an encouraging piece of evidence to pursue future investigation about sex-selective effect of dietary fatty acid.

It is notable that the evidence of detrimental SFD effect on cardiac function is absent, as was evidence of beneficial effect of N3D. Experimentally this matter requires further examination using comparative ex vivo models - it is possible that working heart and Langendorff models offer different functional perspectives. The literature arising from dietary studies using isolated heart models has not yet generated consistent findings (Abdukeyum, Owen, and McLennan 2008; Goo et al. 2014; Pepe and McLennan 2002).

6.3 Conclusion

In conclusion, this study has shown that in context of a low-fat diet, that selective dietary augmentation of the conventional ‘good fat’ i.e. n-3 PUFA; or conventional ‘bad fat’ i.e. saturated fat, had no effect on male and female rat cardiac performance. However, replacement of dietary saturated fat with n-6 PUFA, but not n-3 PUFA, selectively erodes female cardioprotection in ischemia reperfusion. Findings of this study do not contest the current recommendation by National Heart Foundation of Australia to include 2 or 3 fish servings (including oil fish) per week as part of a healthy diet. In terms of n-6 PUFA, despite the detrimental effects reported in the animal component of this study, n-6 PUFA is an essential part of a healthy diet as human do not produce them de novo. However, in light of increasing evidence suggesting potential risks associated with excess n-6 PUFA intake, excessive n-6 intake may be
problematic, especially for women. Rigorous clinical investigations are required to resolve this question.

6.4 References


CHAPTER 7 SUMMARY

The overall goal of this Thesis (submitted with 3 publications) was to examine the effect of ingestion of fatty acid types and classes on cardiac membrane structure and function. A small cohort human supplementation study demonstrated that effective outcome evaluation requires stratification of participants independent of randomization to treatment groups. A stringently controlled rodent diet replacement study demonstrated at the cardiomyocyte and cardiac level that replacement of saturated fat with omega-6 lipid has detrimental effect on female cardioprotection. Considered together, these studies provide useful direction for further investigations of the cardiac impacts of FA-modified diets and supplementation.