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Characterising the omega-3 fatty acid status of the human heart

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CHARACTERISING THE OMEGA-3 FATTY ACID STATUS OF THE HUMAN HEART

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by

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DECLARATION

I, Mandy Lee Theiss, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Masters of Science (Research), in the Department of Biomedical Science, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

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December 2012

"Small privations are easily endured when the heart receives better treatment than the body."

J.J. Rousseau

ACKNOWLEDGEMENTS

On completing this thesis, I have taken stock of the time taken away from the people who have helped me in this endeavour.

First and foremost, I wish to thank Professor Peter L McLennan for his time, encouragement and unwavering patience in helping me complete this tome. Peter, your steadfastness has been an example that I have wished to emulate: there is hope for me yet!

I embrace my friends Ms Zoe Mills, Mr Andrew Frith and Mr Marc Brown who have provided me with the funniest of stories over coffee, the happiest of moments in Wollongong, the broadest (and most absorbent) of shoulders on which to cry, but foremost, without their staunch friendship I would not have progressed pass page one.

I wish to acknowledge the influence of my Nan, Mother and sisters whose intrinsic moral compass has always motivated me to be a better person and to strive for further achievements.

Finally, I also wish to acknowledge the many friends not named here and acquaintances that have wished and urged me on to completion – thank you all.

ABSTRACT

Regular consumption of fish is associated with low morbidity and mortality from acute and chronic cardiovascular disease in humans. Fish are known to contain high concentrations of essential omega-3 (now referred to as n-3) polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA 20:5 n-3) and docosahexaenoic acid (DHA 22:6n-3). Experimental studies modelling cardiovascular disease in animals show that feeding fish oils provides many cardioprotective actions and those directly related to heart function (prevention of fatal cardiac arrhythmias; preconditioning, heart rate slowing, prevention of heart failure) are associated with the incorporation of the diet – derived n-3 PUFA into myocardial membranes. Human red blood cell (RBC) membrane EPA+DHA concentration has been found to correlate inversely with adverse cardiovascular outcomes and is proposed as a cardiovascular risk factor (termed the Omega-3 Index). The use of this readily obtained marker as a risk factor is based on the premise that it reflects the composition of the myocardium at risk. However this is by no means certain as, in animal tissues DHA is incorporated into myocardium well above circulating levels with little EPA detectable, whereas EPA and DHA are better matched within RBC membranes. There are large variations in relative concentrations of n-3 PUFA amongst other tissues in animals and in man. While there is a growing body of data on human atrial tissue obtained during surgical procedures, and blood samples are readily obtained, the limited data on the fatty acid composition of human ventricular myocardium is largely from cadaveric samples. It remains to be confirmed that the omega-3 index can be reliably used as a marker of the composition of the human ventricle and therefore an indicator of risk of cardiac morbidity and mortality. ***Objectives***: To establish the fatty acid compositional profile of human ventricular myocardium and the relationships between EPA+DHA in human RBC, atria and ventricle.

Methods*:* This thesis combined two separate studies in which the fatty acid composition of both atria and ventricles were analysed from fresh, donor hearts and freshly explanted failing hearts, all made available through a heart transplant program, and right atrial biopsy and red blood cells were analysed from subjects undergoing cardiac surgery. Surgical subjects were additionally randomised to receive a daily supplement of 1g fish oil or placebo, commencing at the time of pre-admission check (baseline). Blood

samples were taken at baseline and blood and atrial samples were taken peri-operatively.

***Results**:* DHA was identified as the major n-3 PUFA in all tissues, with concentrations significantly higher than EPA and the n-6 PUFA arachidonic acid (AA) and linoleic acid (LA) were the predominant PUFA (left ventricle: DHA 4.60±1.50; EPA 0.69±0.33; AA 22.95±2.97; LA 20.66±2.55 mean%±SD, N=38). Patterns of incorporation were different in different tissue types however no differences were seen between biopsy samples of right atria and donor right atria in the separate studies. Supplementation with fish oil significantly elevated RBC and atrial biopsy EPA+DHA. Despite variations in proportions of EPA and DHA from tissue to tissue and even greater variations between subjects, the concentration of EPA+DHA always provided the best tissue to tissue correlation within subjects. It was highly correlated between RBC and right atria biopsy (r^2 = 0.4, P=0.0004) and between right atria donor and left ventricle donor (r^2=0.7, P<0.0001). The heart failure study revealed significant increases in DHA in myocardium associated with both age and the stress of heart failure.

Conclusions: This thesis has established that DHA is the main n-3 PUFA in human heart as it is in animal studies, with a range that overlaps with unsupplemented and low-dose supplemented laboratory rat. The DHA concentration is elevated as an apparently compensatory response to stressors and in response to dietary fish oil, as it is in animal studies. With DHA commonly the main n-3 PUFA of table fish, but EPA the principle n-3 PUFA provided as supplements in many clinical trials, establishing the pre-eminent position of DHA amongst n-3 PUFA in human myocardium increases confidence in the consistent human epidemiological studies associating usual fish consumption with cardiovascular outcomes and in translating outcomes of animal studies to interpret mechanisms of n-3 PUFA action in man. It also may provide some explanation for the more variable outcomes of clinical trials.

Despite the consistent predominance of DHA over EPA and variations in their relative concentrations in myocardium and RBC, this thesis has established that the omega-3 index provides the most robust correlations of red blood cells with human heart tissue and can be regarded as a good indicator of myocardial membrane n-3 PUFA composition, confirming its potential as a marker of cardiac-associated risk.

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ABBREVIATIONS

~	approximately
<	less than
=	equal to
^{o}C	degrees Celsius
m*g*	milligrams
g	grams
K*g*	kilograms
μL	microlitre
mL	millilitre
mM	millimolar
w/v	weight per volume
v/v	volume per volume
sn	stereospecific numbering
α	alpha
Δ	delta
ω	omega
Σ	sigma; sum of
AA	arachidonic acid
ACC	American College of Cardiologists
AHA	American Heart Association
ANOVA	analysis of variance
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
CI	confidence interval
CO	cardiac output
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DPA	docosapentaenic acid
EPA	eicsapentaenoic acid
FA	fatty acid
FAME	fatty acid methyl esters
FID-GC	flame ionising detector – gas chromatography

GC	gas chromatograph
HF	heart failure
IUPAC	International Union of Pure and Applied Chemistry
LA	linoleic acid
LSD	least significant difference
M	molar
MI	myocardial infarction
MUFA	monounsaturated fatty acid
NHFA	National Heart Foundation of Australia
NYHA	New York Heart Association
OA	oleic Acid
PA	palmitic acid
PL	phospholipid
PoA	palmitoleic acid
PO_4	phosphate molecule
PUFA	polyunsaturated fatty acid
RBC	red blood cells; erythrocytes
SFA	saturated fatty acid
SA	stearic acid
SCD	sudden cardiac death
SD	standard deviation
SEM	standard error of the mean
SPE	solid phase extraction
SV	stroke volume

CONFERENCE PRESENTATIONS

ORAL PRESENTATIONS

Title: The fatty acid composition of atria and ventricle from healthy donor and explanted (failing) human heart

Authors: Theiss, M. L., McLennan, P.L., Sheeran, F., Pepe, S.

Conference Proceedings: ISSFAL 2006: International Society for the Study of Fatty Acids & Lipids incorporating The 6th International Congress on Essential Fatty Acids and Eicosanoiods and PUFA in Maternal and Infant Health 2006 Annual Scientific Meetings

Conference Year and Location: 2006, Cairns

POSTER PRESENTATIONS

Title: Fatty acid composition of red blood cell membranes as a marker of human heart membrane phospholipid fatty acids

Authors: Theiss, M. L., Pepe, S., McLennan, P. L.

Conference Proceedings: Australian Physiological Society, AuPS

Conference Year and Location: 2005, Canberra

Title: Omega-3 fatty acids in human heart: correlation between atria, ventricle and erythrocytes

Authors: Theiss, M. L., McLennan, P.L., Pepe, S., Sheeran, F.

Conference Proceedings: 5th Annual Conference of the Society for Heart and Vascular Metabolism (SHVM)

Conference Year and Location: 2007, Maastricht, Netherlands

Title: Fatty acid composition of human heart: Setting the baseline

Authors: Theiss, M. L., McLennan, P.L., Sheeran, F., Pepe, S.

Published Source: FROM CELL TO MAN TO SOCIETY, Journal of Molecular and Cellular Cardiology

Conference Proceedings: XIX World Congress of the International Society for Heart Research (ISHR)

Conference Year and Location: 2007, Bologna, Italy

Chapter 1

LITERATURE REVIEW

1.0. INTRODUCTION

The World Health Organisation in 2001 listed cardiovascular diseases as one of the three neglected global epidemics, the greatest killer in developing countries, in decline in Western countries but still a problem, straining government health budgets across the globe (WHO, 2003). Australian records estimate 300,000 people having chronic heart failure (HF) with 30,000 new diagnoses each year and 50% of new HF caused by ischaemic heart disease (IHD) (Krum, *et al.*, 2006). In 2001 the United States had approximately 5 million people with HF and 500,000 persons with newly diagnosed HF each year (ACC/AHA, *et al.*, 2001). Modifiable heart disease risk factors include smoking, hypercholesterolaemia, hypertension, diabetes, sedentary lifestyles, obesity and the emergent importance of the Omega-3 Index (Harris, *et al.*, 2004b); the Index is a measure of red blood cell membrane (RBC) phospholipid fatty acids EPA+DHA (EPA (eicosapentaenoic acid, 20:5n-3[1]) plus DHA (docosahexaenoic acids, 22:6n-3)) concentrations. Harris and von Schacky have identified an Omega-3 Index of less than 4% to signify a greater risk for cardiovascular diseases (CVD) death and an Omega-3 Index greater than 8% with the lowest risk of CVD death.

Correlations between red blood cell EPA+DHA and human myocardial EPA+DHA has been difficult to obtain; the myocardial biopsy has been taken during corrective cardiac surgery for valve replacement and /or coronary artery bypass from the right atria (Garg, *et al.*, 2006; Metcalf, *et al.*, 2007) or the right interventricular septum at a 6-month post-heart transplantation check-up for rejection (Harris, *et al.*, 2004a). However, these samples were obtained from disease affected hearts, either heavily influenced by anti-

[1] n-, is the current IUPAC notation used to describe the location of the first double bond in the carbon chain from the terminal or omega (ω) end of the particular fatty acid of interest. Fatty acids are designated by chain length : number of double bonds.

rejection drugs or from a life-time of cardiac disease. There is a lack of evidence to the nature of myocardial membrane phospholipid fatty acid composition in the disease-free human heart furthermore; there is a lack of evidence profiling all four chambers of the myocardium from viable normal myocardium or a failing heart. While there is some data correlating red blood cell fatty acid composition to heart via atria, there is no data validating the relationship between atria and ventricles. It is principally the ventricle that is of interest in HF or as substrate for fatal cardiac arrhythmias.

To confidently utilise the Omega-3 Index as a risk factor for CVD, the fundamental question needs answering as to whether the Index can be used to correctly reflect EPA+DHA concentrations in membrane phospholipid fatty acids found in normal hearts and failing hearts. This thesis will determine the reliability of using the right atrial biopsies for EPA+DHA concentration comparisons with RBC EPA+DHA. Further this thesis will fill in the gaps of knowledge regarding the membrane phospholipid fatty acid composition of all four chambers of the human heart, both failing and normal. In so doing it will determine the reliability of atrial samples reflecting the ventricle fatty acid composition.

1.1. DIET AS A FACTOR MODULATING MEMBRANE FATTY ACID COMPOSITION

Humans and other mammals lack the metabolic capacity to manufacture polyunsaturated fatty acids *de novo*, therefore if needed, they must be obtained in the diet (Clandinin*, et al.*, 1991; Clandinin, 1997). Early last century, Burr established the essentiality of dietary fats (linoleic acid, 18:2n-6, LA and arachidonic acid, 20:4n-6, AA) in rodents for the prevention of disease (Burr*, et al.*, 1929; Burr*, et al.*, 1930).

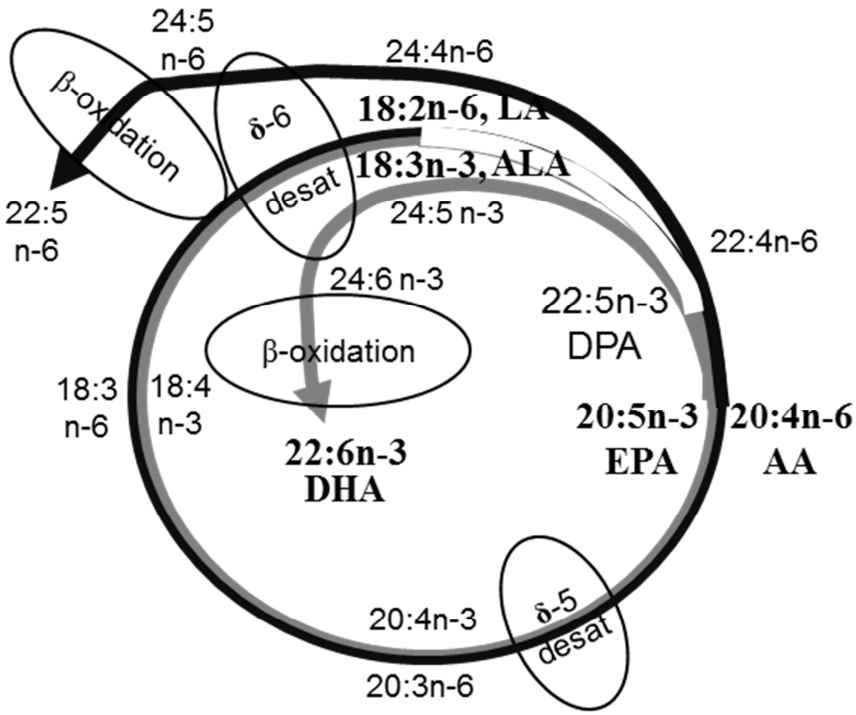

Figure 1.1. Biosynthesis of the omega-3 and omega-6 PUFA.

Grey – n-3 PUFA; Black – n-6 PUFA. Desaturation of medium chain n-3 and n-6 PUFA, ALA and LA, into the long and very-long chain fatty acids, 22:6n-3 (DHA) and 22:5n-6. At several points in the formation of subsequent fatty acids there is competition for the same desaturase enzymes, delta- 6 and delta-5 desaturase (δ-6 desat and δ-5 desat). Omega-3 PUFA are preferentially desaturated (Stubbs*, et al.*, 1984), however the enzymes can be overwhelmed by the amount of n-6 PUFA available in the diet (Holman*, et al.*, 1981). Furthermore, there is limited conversion of ALA to the longer chain n-3 PUFAs, especially DHA in humans (Brenna, 2002). Preformed EPA and DHA in the diets are beneficial to avoid deficiencies (Burdge*, et al.*, 2005).

Today, the essential polyunsaturated fatty acids (EFA) are considered to be the n-6 PUFAs LA and AA (AHA*, et al.*, 2009) and LA is commonly found in terrestrial seeds and grains (Simopoulos, 1991; Gebauer*, et al.*, 2006) of soy, corn and safflower. Arachidonic acid is readily formed from LA (Figure 1.1), but there is no interconversion between n-6 PUFA and n-3 PUFA, which must be obtained separately from the diet. The n-3 PUFA alpha-linolenic acid (ALA; 18:3n-3) can be derived from terrestrial plants (linseed, soy canola) and EPA and DHA consumed as fish. Alpha-linolenic acid

conversion to EPA or DHA in humans is limited (Brenna, 2002). One factor which can be argued is the desaturase enzymes (delta-6 and delta-5 desaturase) being overwhelmed by n-6 PUFA in the diet (Clandinin, 1997; Spector, 1999; Arterburn, *et al.*, 2006; Brenna, *et al.*, 2009), and conversion of ALA to DHA is poor, in part because of the competition between ALA and high basal intakes of LA but also because as ALA in the diet increases, it will join with LA to compete with the intermediate 24:5 n-3 for the delta-6 desaturase conversion to 24:6 n-3 and ultimately DHA (22:6 n-3) (Figure 1.1). In contrast, one important animal study (Slee, *et al.*, 2010) has shown rats consuming human equivalent amounts of n-3 PUFA as FO, despite background diets high in n-6 PUFA, are able to incorporate DHA into myocardial membranes unimpeded. Support for preformed DHA consumption is strengthened (Jump, 2002; Burdge, *et al.*, 2005; Griffin, 2008; Barceló-Coblijn, *et al.*, 2009; Brenna, *et al.*, 2009) and as shown for cardiovascular health, the type of polyunsaturated fat is more important than the amounts of fat in the diet (Hu, *et al.*, 2001; Cordain, *et al.*, 2005). Current dietary recommendation from the American Heart Association calls for 5-10% of daily energy intake from n-6 PUFA (AHA, *et al.*, 2009) with 1-2% of daily energy intake from n-3 PUFA (WHO/FAO, 2004); equalling 1.5-3 *g* per day of ALA and 0.5-1.8 *g* per day of EPA and DHA (AHA, *et al.*, 2002). The usual intake of the Australian diet falls well short of that target (Meyer, *et al.*, 2003) (see Appendices A & B).

1.2. HUMAN STUDIES

Alterations to the heart's structural constituents can influence the heart's electrophysiology; the functioning of ion channels and pumps, and can alter blood flow

to the myocardium causing ischaemia and infarction. These myocardial electrical disturbances can possibly initiate heart failure characteristics and fatal arrhythmias.

Early associations linking diets to decreased heart disease deaths was seen in Norwegian families during the Second World War (Strom, 1948; Strom*, et al.*, 1951). Nelson found coronary heart disease patients lived longer (an extra 51 months) than controls on a diet over 16-19years who included fish, at least 5 servings per week (Nelson, 1972). The Framingham Study (Massachusetts, U.S.A.) (Dawber*, et al.*, 1951; Dawber*, et al.*, 1957) reported high blood cholesterol levels as a significant risk factor in morbidity and mortality in CVD and influentially directed the diet agenda of reducing saturated fat and cholesterol containing foods in the diet for prevention and treatment of heart disease in humans; with the addition to pharmacological measures.

Dyerberg and Bang's observations of the low occurrence of ischaemic heart disease amongst Inuit populations despite diets high in fat (Dyerberg*, et al.*, 1975, 1989) came during the 1970's. Subsequent diet analysis identified the Inuit diet, sourced largely from the sea, as being rich in the long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), EPA and DHA (Bang*, et al.*, 1980; Dyerberg, 1986) and one line of interest focused on the identification of EPA as integral to the formation of the eicosanoids, thromboxane-A3 (TXA_3) and prostacyclin-I3 (PGI_3); the 3-series being responsible for the reduced incidence of platelet aggregation and thrombus formation (Dyerberg*, et al.*, 1978a; Dyerberg*, et al.*, 1978b), with PGI_3 acting upon the vascular endothelium by stimulating a moderate vasodilatory action (Needleman*, et al.*, 1979) in the prevention of CVD. It was shown that Inuit diets were high in cholesterol when compared with Danes (Table 1.1); comparable total fat intakes were seen also, despite less CVD deaths (Bang*, et al.*, 1971; Dyerberg*, et al.*, 1975; Bang*, et al.*, 1976; Bang*, et al.*, 1980; Dyerberg*, et al.*, 1982; Dyerberg*, et al.*, 1989).

Table 1.1. Comparison of EPA and DHA[1, 2] and cholesterol[2] in Inuit and Danish diet.

Fatty Acids	Greenland Inuit (Eskimos)	Danes
EPA[3]	4.6	0.5
DHA[3]	5.9	0.3
Total PUFA[3]	19	13
Omega-3 (*g*/day)	14	3
Omega-6 (*g*/day)	5	10
Cholesterol (*g*/day)	0.70	0.42

EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3). [1] Bang and Dyerberg, 1980; Dyerberg, *et.al.*, 1989. [2] Simopoulos, 2002. [3] Values are % of total fatty acids, except where indicated.

Several longitudinal studies found inverse dose-response relationships to a reduction of coronary heart disease deaths, with as little as 30-40 *g* of fish per week in the 20-year Zutphen study, (Kromhout*, et al.*, 1985). Long-term consumption compared to short-term consumption of fatty fish, follow-up Zutphen study found a significant inverse relationship and sudden cardiac death (SCD) independent of the total amount of fish consumed (Streppel*, et al.*, 2008). Clinically SCD is defined as "the unexpected natural death due to a cardiac cause within a short time period from the onset of symptoms in a person without any prior condition that would appear imminently fatal. It is most often due to a sustained ventricular tachyarrhythmia" (Reynolds*, et al.*, 2010). Shekelle and colleagues re-examined their 25-year the Western Electric Study and found agreement with Kromhout's significant inverse association of fish consumption and reduced risk of death from coronary heart disease (Shekelle*, et al.*, 1985). The US Physicians Health Study found that one serving of fish per week is preventative for SCD via its antiarrhythmic effects on the heart (Albert*, et al.*, 1998). Albert and colleagues also noted an inverse association to whole blood n-3 PUFA and fish intake n-3 PUFA and reduced risk for primary cardiac arrest death (Albert*, et al.*, 2002).

Using RBC membrane phospholipid fatty acids EPA plus DHA as a biomarker of dietary intakes of seafood, Siscovick and colleagues of Seattle identified an inverse relationship to reductions in SCD (Siscovick*, et al.*, 1995). Results indicated that a modest dietary amount of seafood, equal to one fatty fish meal per week or 5.5g of n-3 PUFA per month, was associated with a 50% reduction in primary cardiac arrest. RBC EPA+DHA equal to 5% of total fatty acids were associated with a significant 70% reduction in risk of primary cardiac arrest when compared to EPA+DHA 3% of total fatty acids. Extending this study further, Siscovick highlighted that increasing fish consumption beyond the modest amount of 5.5g EPA+DHA per month would not offer a further reduced risk for primary cardiac arrest (Siscovick, et al., 2000).

In light of the then current animal research indicating antiarrhythmic properties of EPA and DHA (McLennan*, et al.*, 1988) reduced mortality from all-causes in recovered MI patients identified the secondary protection benefits of introducing two or three fatty fish meals per week in the Diet and Reinfarction Trial (DART) (Burr*, et al.*, 1989). The DART trial highlighted a clear separation between fish advice and no fish advice groups at approximately 55-65 days (Figure 1.2); an argument for the initial delay from the beginning of the trial to the separation of survival curves can be ascribed to a time lag in which EPA and DHA can be adequately incorporated into the phospholipid membranes of myocytes to affect cardioprotective benefits as described in later animal studies (Owen*, et al.*, 2004).

The Italian, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico Prevenzione trial (GISSI-P) is the second most significant secondary prevention post-MI trial (Marchioli*, et al.*, 1999). The GISSI-P clearly established from a daily supplement of approximately 850 mg EPA and DHA per day relative risk for cardiac death, coronary death and SCD were significantly reduced. There was no change in

other deaths or non-fatal CV events however. Later time course analysis found early benefits for consuming n-3 PUFA supplements, as survival curves significantly diverged at 90 days for total mortality and at 120 days for SCD (Figure 1.3) (Marchioli, 2002). The GISSI-HF trial (2008) saw symptomatic, chronic heart failure patients benefiting from additional treatment with FO (also approximately 850 mg EPA and DHA) daily in addition to their usual care, although the time course for benefits in was approximately 2 years (Tavazzi, *et al.*, 2008b).

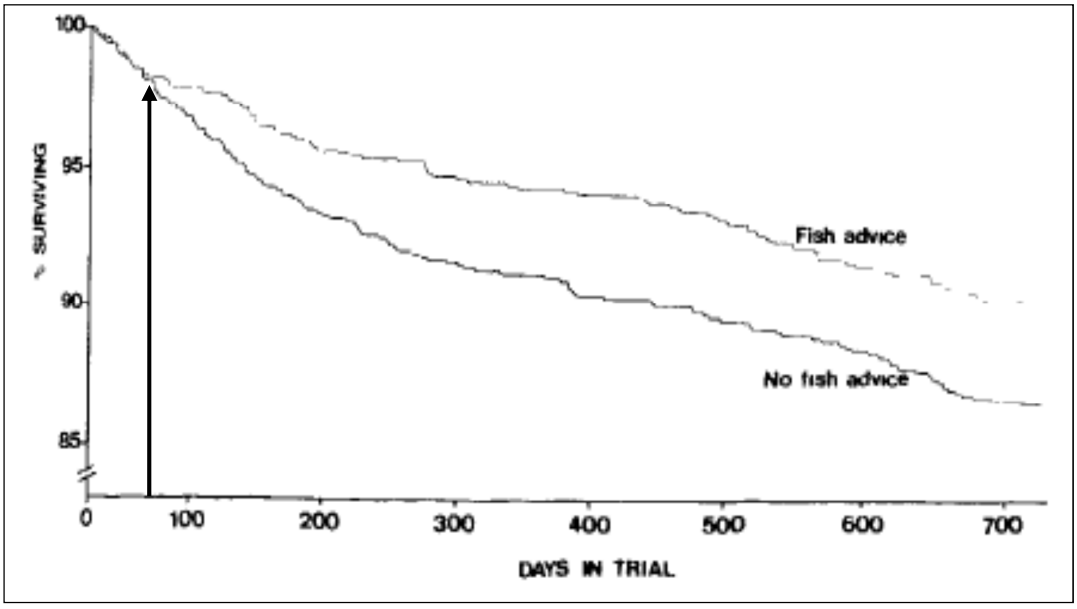

Figure 1.2. Increase in % survival from fish advice over a 2 year period in the DART study.

Fish advice (upper trace) versus no fish advice (lower trace). The arrow indicates the time at which dietary advice separation is seen in survival curves; approximately 55 - 65 days. Taken from DART study of Burr, *et al*., 1989.

Cardiac haemodynamics (Mozaffarian, 2007) and electrographic parameters (Mozaffarian, *et al.*, 2006b) are shown to be improved after dietary fish consumption or FO supplementation; with DHA demonstrated as the bioactive fatty acid (Grimsgaard, *et al.*, 1998; Mori, *et al.*, 1999). Modest reductions are seen in blood pressure (Morris, *et al.*, 1993; Mori, *et al.*, 1999; Mozaffarian, *et al.*, 2006a), heart rates (HR) (Mori, *et*

al., 1999; Dallongeville*, et al.*, 2003; Mozaffarian*, et al.*, 2005b), slower atrioventricular conduction and reduced longer QT intervals (Mozaffarian*, et al.*, 2006b). Increased significant inverse relationship with DHA incorporation into RBC phospholipids (Mozaffarian*, et al.*, 2008). In healthy volunteers increased heart rate variability (HRV) in healthy men without known heart disease, have shown a positively associated with serum cholesterol ester DHA (Brouwer*, et al.*, 2002) and post-MI patients, FO supplementation increased HRV also (Christensen*, et al.*, 1997; Christensen, 2011). However, a small study of selected human trials have shown no effect of n-3 PUFA on various cardiac parameters (Geelen*, et al.*, 2004).

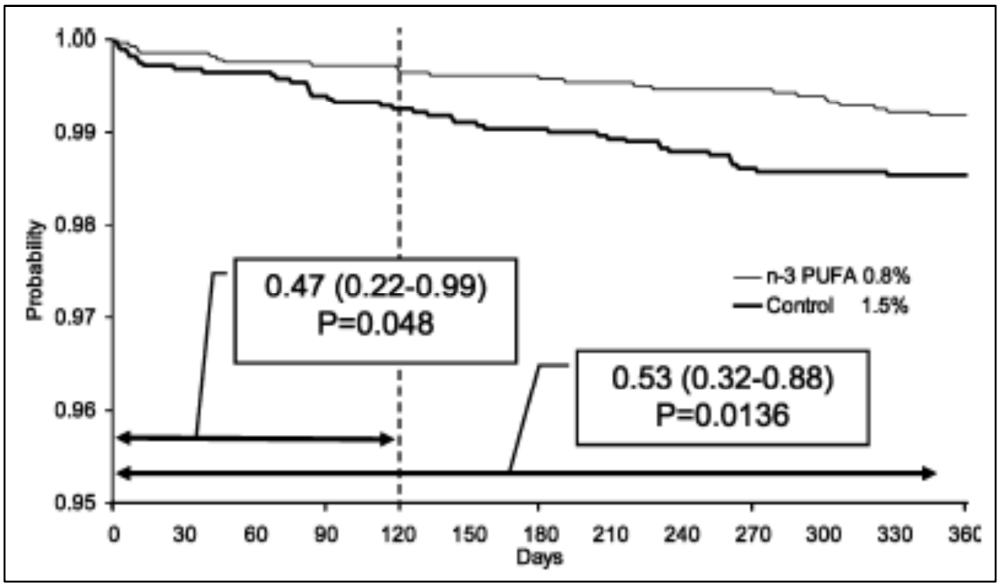

Figure 1.3. Increase in % survival from fish advice over a 3.5 year period from the 2002 GISSI-P study.

Sudden death reduced in 120 days when compared with controls. The arrows along the horizontal axis indicates the time (in days) at which the probability of increases in survival are significantly demonstrated. Taken from GISSI-P Trial (Marchioli, 2002).

Lower concentrations of n-3 PUFA, especially DHA, were identified in association with greater numbers of ventricular arrhythmias in patients with known potential fatal arrhythmias and implanted cardioverter devices (ICD) (Christensen*, et al.*, 2005). The

prospective trial, The Study on Omega-3 Fatty Acids and ventricular Arrhythmia (SOFA) indicated no significant differences between FO or control groups for the end-points of appropriate ICD pacing or death. This trial found however, that patients with a prior MI and taking FO did trend towards longer event-free survival (Brouwer*, et al.*, 2006b). Further, a subset of patients with ICD from the GISSI-HF trial, taking an oral n-3 PUFA supplementation found a 20% reduction in activated ICD discharges; this result was non-significant however (Finzi*, et al.*, 2011). This line of investigation in the effectiveness of co-treatment of patients with ICDs and n-3 PUFA is yet to be thoroughly explored.

Atrial fibrillation (AF) occurs commonly in older individuals of sixty-five years and more, occurring in about 5% of the Australian population (NHFA, 2008a), however after cardiothoracic surgery, there is almost a 50% presentation after valvular surgery and AF develops in 11 to 40% of patients after coronary artery bypass-grafting (CABG) (Ommen*, et al.*, 1997). A lesser number of patients with AF presentations are due to MI. Atrial fibrillation can be added to the story of arrhythmias moderated by EPA and DHA (Mozaffarian*, et al.*, 2004; Reiffel*, et al.*, 2006), however this is contested by others (Frost*, et al.*, 2005; Brouwer*, et al.*, 2006a; Saravanan*, et al.*, 2009). Due to its potential for significant adverse events, recurrent or persistent AF is treated prophylactically with pharmacological preparations (β-blockers, calcium-channel blockers, anti-inflammatory and others) (Crystal*, et al.*, 2002) or surgical ablation of the most common areas for ectopic generation, for example the pulmonary veins (PV) (Haissaguerre*, et al.*, 1998). Ingestion of FO for one month prior to surgery, resulted in significantly longer effective refractory periods than controls in the left and right superior PV and the left atrium, resulting in reduced susceptibility to PV-initiated AF (Kumar*, et al.*, 2011). Oral intakes of tuna or other broiled or baked fish has been associated with lower incidence

of AF and improve atrial function in older adults (Mozaffarian, *et al.*, 2004). Cell studies have shown isolated atrial myocytes reducing their induced asynchronous contractions when acute administrations of EPA or DHA were given, the mechanism for which has been purported to be the increased fluidity of the plasma membranes of these cells (Jahangiri, *et al.*, 2000). The efficacy of pre- and post-operative prophylactic administration of EPA and DHA has been demonstrated in cardiac patients undergoing CABG surgery with 15% of patients developing post-operative AF, compared to 33% in the control group (Calò, *et al.*, 2005). However, several recent studies have failed to observe any benefit of fish oil supplementation in preventing post-operative AF (Saravanan, *et al.*, 2009; Heidarsdottir, *et al.*, 2010; Farquharson, *et al.*, 2011).

Docosahexaenoic acid is found in large concentrations in the membranes of the brain and retina (Arterburn, *et al.*, 2006) and is yet to be fully described in viable human myocardium. The six double bonds in DHA provides a flexibility unmatched by other fatty acids, and so disrupts cholesterol molecules from lateral packing within the membrane bilayer construction and their protein interactions (Stillwell, *et al.*, 2003). Previously, human myocardial membrane phospholipid fatty acids have been generalized from feeding studies in animals.

The last decade has provided numerous scientific reviews detailing positive associations of fish and/or FO supplementation with significant benefits from heart disease and death (Leaf, *et al.*, 2003; Calder, 2004; Harris, *et al.*, 2008b; Lee, *et al.*, 2008; Duda, *et al.*, 2009; Lavie, *et al.*, 2009; Von Schacky, 2009). Alternatively, several authors have found no associations of beneficial effects from dietary fish or FO (EPA and DHA) supplementation on the heart (Ascherio, *et al.*, 1995; Morris, *et al.*, 1995; Nakamura, *et al.*, 2005; Hooper, *et al.*, 2006). Importantly however, several science advisories from the U.S. (Kris-Etherton, *et al.*, 2002; London, *et al.*, 2007), Australia (NHFA, 2008c)

and the United Kingdom (SACN, 2008), in addition to dietary guidelines from governmental agencies (NHMRC, 2003; USFDA, *et al.*, 2010) have supported the consumption of fish and FO supplementation for heart health.

1.3. ANIMAL STUDIES

Animal heart studies clarified the relationships by which dietary fats altered myocardial membranes, thus effecting change in myocardial membrane function. Ready modification of myocardial membranes by a 10% dietary cod liver oil (CLO) in rats was first reported by Gudbjarnason and Oskarsdottir (Gudbjarnason, *et al.*, 1977). The n-3 PUFA, DHA and EPA in CLO were avidly incorporated into membranes, whereas the n-6 PUFA, AA and LA, were significantly reduced over the feeding period (Gudbjarnason, *et al.*, 1977) (Table 1.2). Additionally, DHA was shown to be preferential incorporated into total phospholipids, and then the individual phospholipids of phosphatidylcholine and phosphatidylethanolamine, of noradrenaline-stressed myocardial cell membranes (Emilsson, *et al.*, 1981). Again after CLO diets, fewer rats experienced fatal ventricular fibrillation (VF) induced from repeated β-adrenoceptor stimulation (isoproterenol injections) than controls (Benediktsdottir, *et al.*, 1986). Paradoxically, the endogenous stress of repeated adrenaline injections induced fatal arrhythmias but also increased DHA incorporation in myocardial membranes in rats fed a variety of diets differing in fat types (Benediktsdottir, *et al.*, 1988). Even in CLO fed rats, myocardial membrane compositions shifted to a more dominant DHA composition over and above the diet induced increase and arrhythmias were inhibited (Benediktsdottir, *et al.*, 1988). Upon withdrawal of the stress, the membrane

phospholipid fatty acids returned to near normal baseline compositions (Benediktsdottir, *et al.*, 1988).

Table 1.2. Effect of 10% fat in the diet as cod liver oil[1] compared to a control diet in rats on selected heart membrane fatty acids.

Fatty Acids	Control	Cod Liver Oil	Relative change in composition[2]
LA	30.4	20.6	⇓
AA	13.1	9.4	⇓
DHA	9.3	19.2	⇑⇑

Values are % of total fatty acids. AA, arachidonic acid (20:4n-6); LA, linoleic acid (18:2n-6); DHA, docosahexaenoic acid (22:6n-3). [1.]Gudbjarnason, *et al.*, 1977, 1978. [2] ⇓ - Small decrease; ⇑⇑ - Large increase.

The functional consequences of membrane changes where reviewed by Gudbjarnason (1975) and initially focused on the local cardiac effects of the prostaglandins: increasing myocardial contractility; inhibit endogenous noradrenaline; coronary arterial vasodilation; and antiarrhythmic effects (Gudbjarnason, 1975). The findings of ten Hoor and de Deckere showed linoleic acid from sunflower seed oil increased prostacyclin (PGI_2) release and had positive influences on coronary flow, spontaneous frequency and contractile force in rat (ten Hoor, *et al.*, 1981). At this time the feeding of linseed oil, high in ALA (18:3n-3) had no effect on flow, frequency or force in the rat heart, but did halve prostacyclin production (ten Hoor, *et al.*, 1981).

Abeywardena and colleagues (Abeywardena, *et al.*, 1987) demonstrated in rat heart studies the malleability of the cardiac membranes in dietary cross-over studies. Myocardial membrane phospholipid fatty acids changed significantly to reflect the principal fats in the diet (Table1.3). Thus, sunflower seed oil (SSO) supplying high amounts of LA, increased AA concentrations, and tuna fish oil (FO) saw greater DHA concentrations and suppression of the n-6 PUFA, LA and AA. Agreeing with the earlier

work of Gudbjarnason (1975), DHA is seen to double in concentration from FO feeding with EPA restricted to just detectable concentrations. When supplying large amounts of EPA in the diet, Hock and colleagues (Hock*, et al.*, 1987) still identify DHA as the principal n-3 PUFA in myocardial membranes although EPA concentration is greatly increased. These brief examples highlight that irrespective of the absolute amount of total fat in the diet (12% or 5%), n-3 PUFA diets reduce both n-6/n-3 and AA/DHA ratios, with DHA shown to be the principal n-3 PUFA in myocardial membrane PL fatty acids.

Table 1.3. The effect of different diets on selected myocardial membrane phospholipid fatty acids in rats.

Fatty Acids	Reference Diet[1]	Saturated Fat[2]	High SSO[2]	High FO[2]	Corn Oil[3]	Menhaden Oil[3]
Saturated Fatty Acids						
16:0 (PA)	10.6	9.4	9.3	9.9	12.6	14.6
18:0 (SA)	23.6	24.0	25.6	24.3	22.5	23.4
Monounsaturated Fatty Acids						
18:1n-9 (OA)	7.9	10.2	6.5	8.6	9.07	10.4
Omega-6 Polyunsaturated Fatty Acids						
18:2n-6 (LA)	21.3	13.7	17.8	9.1	20.2	8.2
20:4n-6 (AA)	16.9	18.9	19.3	15.0	23.6	11.5
Omega-3 Polyunsaturated Fatty Acids						
20:5n-3 (EPA)	0.1	0.02	n.d.	0.9	0.1	6.4
22:5n-3 (DPA)	1.5	1.4	0.9	0.7	0.4	3.5
22:6n-3 (DHA)	12.6	17.7	12.8	26.8	3.9	14.9
n-6/n-3 Ratio	2.7	1.7	2.8	0.9	11.1	0.8
AA/DHA	1.3	1.1	1.5	0.6	6.1	0.8

Values are % of total fatty acids. n.d., not detected; Reference Diet, Commercial standard rat chow; SSO, sunflower seed oil; FO, tuna fish oil; 16:0 (PA) palmitic acid; 18:0 (SA) stearic acid; 18:1 (OA) oleic acid;18:2n-6 (LA) linoleic acid; 20:4n-6 (AA) arachidonic acid; 18:3n-3 (ALA) alpha-linolenic acid; 20:5n-3 (EPA) eicosapentaenoic acid; 22:5n-3 (DPA) docosapentaenoic acid; 22:6n-3 (DHA) docosahexaenoic acid. [1] Charnock *et al.* 1986. [2] Abeywardena *et al.* 1987. [3] Hock *et al.* 1987.

McLennan in 1985 reported that modification of myocardial membranes, with dietary PUFA, sunflower seed oil, altered rat heart function to be significantly anti-arrhythmic in an *in vivo* model of MI (McLennan*, et al.*, 1985). Extending the previous experiments to whole animal model of arrhythmia and SCD, McLennan applied coronary vessel occlusion (ischaemia) and reperfusion in tuna FO fed rats and significantly averted VF in both conditions, however sunflower seed oil diets were effective against ischaemic arrhythmias only (McLennan*, et al.*, 1988). Billman and colleagues later confirmed McLennan's significant findings that FO prevented VF from occurring in exercising dogs with a previous MI. In this experimental model, the dogs were not pre-fed EPA and DHA, but an intravenous infusion of EPA and DHA was used (Billman*, et al.*, 1994). However, there is a high probability that the antiarrhythmic mechanisms for dietary incorporated n-3 PUFA and acutely administered n-3 PUFA are not the same (as summarised in (McLennan, 2004)). For example the infusion of n-3 PUFA in the dog is accompanied by a large fall in heart rate, marked ECG changes and a high incidence of heart block (Billman*, et al.*, 1994) suggesting effects on electrical conduction pathways that are not evident after dietary intervention. Most recently the dog was revealed to be highly resistant to incorporation of DHA into myocardial membranes (Billman*, et al.*, 2012) (supplementary data) compared to rat or man, even after extremely high fish oil supplementation, and may not be a good model for investigation of n-3 PUFA dietary actions. Jordan and others, in a report for the U.S. Department of Health and Human Services, summarised the experimental data and identified pre-fed FO in whole animals (rats) as being more effective than n-6 PUFA in the prevention of ischaemia-induced VF, and especially reperfusion-induced VF and total arrhythmic deaths. When compared to pre-fed monounsaturated fats, DHA alone or EPA plus DHA significantly reduced VF incidence; EPA alone did not significantly lower VF (Jordan*, et al.*, 2004).

An important development in cardioprotection from MI is that of ischaemic preconditioning (IPC) (Murry, *et al.*, 1986). An alternate to pharmacologic preconditioning induction, Abdukeyum from the McLennan laboratory utilised a high-DHA dietary supplement in rats to affect the protectiveness of IPC; limiting infarct size, lethal myocardial cell injury and arrhythmia (Abdukeyum, *et al.*, 2008). In whole animal ischaemia-reperfusion studies, the myocardial membrane property of electrical excitability/vulnerability is altered after different dietary fat feeding (saturated, n-6 and n-3 diets) thence, altered membrane compositions (McLennan, *et al.*, 1989). Fish oil feeding leading to changes in membrane phospholipid fatty acids produces functional benefits in rat hearts including anti-arrhythmic effects (McLennan, *et al.*, 1988; Pepe, *et al.*, 1996), reduced fibrillation and sudden cardiac death after reperfusion (McLennan, *et al.*, 1989), reduced myocardial injury and improved contractility following ischaemia and reperfusion (Pepe, *et al.*, 1996; McLennan, 2001; Pepe, *et al.*, 2002), lower resting heart rates (McLennan, *et al.*, 2005) and in female mice, hypertrophy is suppressed in pressure-overloaded hearts (Huggins, *et al.*, 2009). These animal studies are in accord with Gudbjarnason (Gudbjarnason, 1975) where preferential increases in membrane DHA corresponded to reductions in n-6 PUFA arachidonic acid (AA, 20:5n-6) and linoleic acid (LA, 18:2n-6), though not proportionally. Other functional benefits to the heart in animals from dietary n-3 PUFA include attenuation of cardiac hypertrophy (Huggins, *et al.*, 2009) and reducing myocardial oxygen consumption (Pepe, *et al.*, 2002).

In 2006, Reiffel highlighted the disparities in methodology of human clinical trials and the inconsistency with which the results of these trials provide doubt towards the antiarrhythmic effectiveness of dietary EPA and DHA, and questions whether the type of dietary oil mattered (Reiffel, *et al.*, 2006). It has been observed that the n-6 PUFA

antiarrhythmic actions were lost when mixed with saturated fat (Charnock*, et al.*, 1991; McLennan*, et al.*, 1993). Importantly for translation to humans, Slee (2010) has reported that on very low intakes, comparable to human dietary recommendations of 1-2 fish meals per week, FO impacts significantly on the incorporation of DHA into myocardial membrane phospholipid fatty acids in the face of a high n-6 PUFA background diet (Slee*, et al.*, 2010). This study supports the previous reports of low doses of dietary FO effecting myocardial membrane changes in DHA and EPA. Further, Owen and colleagues (Owen*, et al.*, 2004) found the myocardium of rats required only 2 days for significant changes to be observed in n-3 PUFA incorporation into the membranes of the myocardium, particularly DHA, with a maximal effect achieved after 4 weeks. RBC membranes achieved significant changes in DHA and EPA after 4 weeks (Owen*, et al.*, 2004). Significantly, arrhythmia prevention was not evident until at least 7 days of feeding (McLennan 2001 Lipids), and isolated heart studies demonstrate that membrane incorporation rather than circulating fatty acids provides arrhythmia prevention (Pepe*, et al.*, 1996; Abdukeyum*, et al.*, 2008).

Different animal species exhibit different myocardial membrane PL fatty acids compositions (Table 1.4). Notably whole hearts, ventricles and atrium display differences in membrane PL fatty acid composition, however, it can been seen that the fatty acid profiles demonstrate similarities such as DHA as the principal n-3 PUFA in the whole heart, ventricles and atrium with the exception of the guinea pig heart. Alpha-linolenic acid (ALA, 18:3n-3) and EPA are both found to be close to undetectable, in many cases they are not reported. The n-6 PUFA are a significant proportion of total fatty acids. There is a trend for LA to be greater in concentration than AA in the ventricles of these animals shown here in Table 1.4. In the atrium AA is greater than LA. Differentiations between the left side of the heart and the right side cannot be

shown, which would shed light upon the functional differences imposed on the left and right ventricles.

Different diets exhibit different myocardial membrane PL fatty acids compositions in rats (Table 1.5). Notably 12% tuna FO more than doubles (the initially substantial) DHA concentration in the whole heart and left ventricle, with 5% menhaden oil increasing (initially very low) EPA by 32 times and marginally increasing DHA. The n-6 PUFA, LA and AA, are suppressed in the tissues by FO and menhaden oils. When diets high in n-6 PUFA, 12% sunflower seed (SSO) and 5% corn oils (CnO), are fed to rats LA and AA are more evenly distributed when compared to a standard chow diet which sees LA to be almost double that of AA. Corn oil reduces LA and increases AA to near equal concentrations also, but to concentrations higher than SSO. A saturated fat diet reduces LA and conserves AA, it also increases DHA and EPA is reduced to barely detectable.

1.4. MYOCARDIAL MEMBRANE PHOSPHOLIPID FATTY ACIDS OF THE HUMAN HEART

At autopsy, from the hearts of deceased persons whom died of sudden cardiac death (SCD) (5 out of 8 cadaveric male human hearts) Gudbjarnason and Hallgrimsson first identified a doubling of membrane DHA, with LA and AA reduced up to a third (Gudbjarnason*, et al.*, 1975a). To date a comprehensive presentation of the composition of the human cardiomyocyte membrane phospholipid fatty acids remains elusive, principally due to the difficulties in obtaining healthy viable tissue samples for analysis. In the past twenty-five years there have been some reports of heart muscle lipid analyses however, these heart samples have been associated with valve disease (Rocquelin*, et al.*,

1985; Rocquelin*, et al.*, 1989; Metcalf*, et al.*, 2007), cardiac transplantation (Harris*, et al.*, 2004a), cardiomyopathy (Belfrage*, et al.*, 1979), mitochondrial and microsomal fractions (Gloster*, et al.*, 1969, 1970), coronary artery bypass grafting surgery (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010) and cadavers (Simon*, et al.*, 1969; Fletcher, 1972; Gudbjarnason*, et al.*, 1975a; Gudbjarnason*, et al.*, 1975b; Heckers*, et al.*, 1977; Gudbjarnason*, et al.*, 1978c; Belfrage*, et al.*, 1979; Ray*, et al.*, 1979; Gudbjarnason, 1980; Shenolikar, 1980; Ohlrogge*, et al.*, 1981; Sen*, et al.*, 1981; Sexton*, et al.*, 1995). These analyses are assumed to be the standard for the human heart, however some samples are problematic, in particular the cadaveric samples due to the varying times after death that these tissue samples have been harvested. Upon death there is the loss of membrane integrity with autolytic changes occurring, with the amount of cell damage and loss of fatty acids unknown (Gudbjarnason*, et al.*, 1978c).

Systematic phospholipid fatty acid analysis of each chamber of the heart has not been carried out in the human heart. The variety of analytical methods used to present each of the above works, does not present a coherent story for today. The variety of results therefore is reflected here by the limited numbers of fatty acids identified in some papers seemingly for technical reasons (Gloster & Harris, 1969; Fletcher, 1972), molecular phospholipid species identified (Gloster & Harris, 1970; Fletcher, 1972; Ray & Sengupta, 1979; Sen & Sengupta, 1981), microsomal and mitochondrial results (Gloster & Harris, 1969; Fletcher, 1972), very small sample numbers (n=1 or 2) (Simon & Rouser, 1969; Gudbjarnason & Halgrimsson, 1975; Belfrage, 1979) and perplexing results from a very small intraventricular catheter biopsy (questionable sample type – possibly epithelium not muscle tissue) (Harris *et al.*, 2004).

Anticipating the questions of relevance to the human condition, Gudbjarnason and

Hallgrimsson provided phospholipid fatty acid data on several human hearts. Cadaveric hearts, with deaths from SCD and MI, were to provide evidence of the same inverse relationship of DHA to n-6 fatty acids as that found in their rat experiments. Further relationships garnered from these results saw that increasing age brought increases in myocardial membrane DHA (Gudbjarnason, 1980) and males were found to have higher membrane DHA than females. The ratio of AA/DHA is proposed as a risk for SCD and DHA is required for normal heart rate control (Gudbjarnason*, et al.*, 1978c).

There has been one study that has given an understanding of the differences in fatty acid concentrations in the atria and the ventricles of the rat heart (Charnock*, et al.*, 1983). There are no studies to date which report the membrane fatty acid composition of normal human hearts, moreover, there are no studies that have differentiated the regional differences in fatty acid composition between each atria or each ventricle of the human heart.

Red blood cell membrane fatty acid, plasma and serum EPA and DHA are very good at indicating a short term dietary profile. The Omega-3 Index which is the sum of EPA and DHA in the membranes of red blood cells (RBC EPA+DHA), is proving valid for the status of EPA+DHA in the human heart. An elevated Omega-3 Index, equal to or more than 8.0 per cent is purported to be valid for heart health (Harris*, et al.*, 2004b). Most recently, a low Omega-3 Index has been associated with protection against VF after sudden cardiac arrest (Aarsetoey*, et al.*, 2011). The studies presented in this thesis will show evidence for the normal composition of human heart phospholipid fatty acids, show the close correlation between human red blood cell membrane phospholipids fatty acids and human right atrium phospholipid fatty acids, and give evidence for the correlation between red blood cell membrane fatty acids and human left ventricle membrane fatty acids, particularly, the Omega-3 Index and left ventricular EPA+DHA.

Table 1.4. Heart Membrane Phospholipid Fatty Acid Composition from Different Animal Species Consuming a Control Diet.

Fatty Acids	[1]Rat Heart	[2]Rat Atrium	[2]Rat Ventricle	[3]Rat Left Ventricle	[4]Marmoset Atrium	[4]Marmoset Ventricle	[5]Guinea Pig Heart	[6]Mouse Ventricle	[7]Pig Heart	[8]Dog Heart	[9]Cat Right Ventricle
N=	12	13	11	11			12		7	5	5
18:2n-6 (LA)	26.7	13.5	24.9	21.3	15.0	21.8	24.6	16.3	6.1	25.2	30.9
20:4n-6 (AA)	15.4	21.1	15.9	16.9	18.0	16.9	23.9	10.6	3.1	28.3	7.5
18:3n-3 (ALA)	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.r.	n.r.	0.1	n.r.	n.r.
20:5n-3 (EPA)	0.2	n.d.	n.d.	0.1	n.r.	n.r.	n.r.	n.d.	0.2	n.r.	n.r.
22:5n-3 (DPA)	1.5	1.3	1.0	1.5	2.3	2.0	0.8	n.r.	0.3	n.r.	n.r.
22:6n-3 (DHA)	12.4	8.3	12.3	12.6	3.5	3.4	0.3	24.2	0.3	n.r.	1.4

Values are % of total fatty acids. n.d., not detected; n.r., not recorded; 20:4n-6 (AA), arachidonic acid, 18:3n-3 (ALA), alpha-linolenic acid; 22:6n-3 (DHA), docosahexaenoic acid; 20:5n-3 (EPA), eicosapentaenoic acid; 18:2n-6 (LA), linoleic acid; 18:1 (OA), oleic acid; 16:0 (PA), palmitic acid; 18:0 (SA), stearic acid. [1]Gudbjarnason, *et al.*,1978. [2]Charnock, *et al.*,1983. [3]Charnock, *et al.*,1985. [4]Charnock, *et al.*,1985. [5]Abedin, *et al.*,1999. [6]Huggins *et al.*,2009. [7]Nair *et al.*,1999. [8]Van der Vusse *et al.*,1982. [9]Reibel *et al.*,1986.

Table 1.5. The Effect of Different Diets on Heart Membrane Phospholipid Fatty Acids in Rats.

Fatty Acids	Saturated Fat[1]	High SSO[1]	High FO[1]	High SSO[2] LV	High FO[2] LV	Corn Oil[3]	Menhaden Oil[3]
16:0	9.4	9.3	9.9	9.4	10.8	12.6	14.6
18:0	24.0	25.6	24.3	26.6	26.4	22.5	23.4
18:1	10.2	6.5	8.6	5.6	6.7	9.07	10.4
18:2n-6 (LA)	13.7	17.8	9.1	18.0	8.3	20.2	8.2
20:4n-6 (AA)	18.9	19.3	15.0	19.1	13.1	23.6	11.5
20:5n-3 (EPA)	0.02	n.d.	0.9	n.d.	1.3	0.1	6.4
22:5n-3 (DPA)	1.4	0.9	0.7	1.0	n.d.	0.4	3.5
22:6n-3 (DHA)	17.7	12.8	26.8	11.2	26.8	3.9	14.9

Values are % of total fatty acids. n.d., not detected; LV, left ventricle; SSO, sunflower seed oil; FO, tuna fish oil; AA, arachidonic acid; ALA, alpha-linolenic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. [1]Charnock *et al.*,1985. [2]Charnock *et al.*,1986. [3]Hock *et al.*,1987.

1.5. RESEARCH CONSIDERATIONS

As a model moves away from the clinical picture of human heart disease, researchers need to balance five elements to complete a study; those elements include expertise, time, cost, relevance and data/results (Figure 1.4). Heart research is dependent upon the insights gained from animal studies however the principal weakness of animal experiments is the direct extrapolation to the human condition. Numerous animal models have been developed to explore the many facets of heart disease, such as heart failure and hypertrophy. In the four year period 1993-1997, a total of 1943 papers on heart failure and hypertrophy were produced (see review by Hasenfuss, 1998) utilising numerous models of the disease and numerous animals; these included 18 different types of rat model equaling 1028 papers, 6 rabbit, 11 dog, 3 pig, 2 guinea pig, 3 mouse,

2 cat, 2 hamster, 1 turkey, 1 ferret, 3 sheep, 2 baboon and 1 bovine model, as well as 20 transgenic animal models (Hasenfuss, 1998).

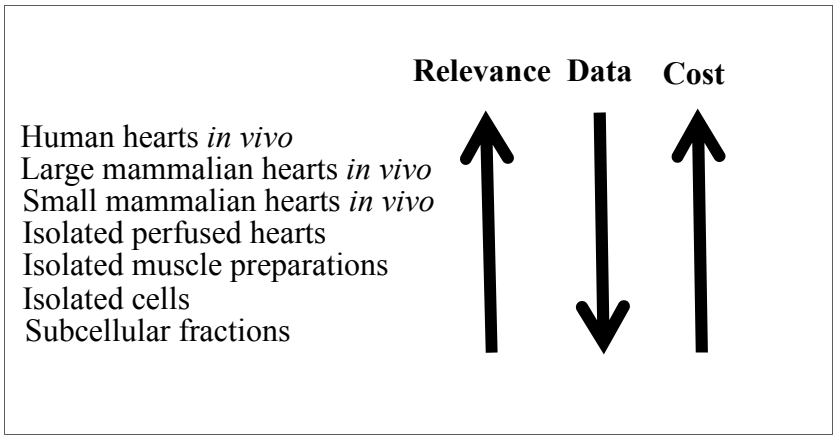

Figure 1.4. The compromises of cost and clinical relevance versus experimental value when investigating human heart disease.

This scheme identifies the difficulty of maintaining focus on research specificity when studying the mechanisms of human heart disease. Diagram taken from Hearse and Sutherland (2000).

1.6. AIMS AND SIGNIFICANCE

The raft of information that is available from animal studies and the epidemiological results give a clear indication that the n-3 PUFA, especially DHA, may be important for a healthy and optimal heart function. Myocardial membranes of human hearts have been investigated in previous studies using either cadaveric tissue or small atrial biopsy samples but direct comparisons of phospholipid fatty acids from the atria and ventricles is lacking.

There has been no clear evaluation of healthy human heart composition or comparison of human failing and non-failing hearts to aid interpretation of epidemiological data or to help establish the validity of experimental animal models.

Aim 1a: *To identify the major fatty acids in the healthy human heart to set the baseline for investigation of relationship of fatty acid composition to heart function in health and disease.*

Aim 1b: *To compare fatty acid composition of all heart chambers to establish the validity of opportunistic atrial sampling as a marker of human heart composition.*

Aim 1c: *To compare healthy and failing human heart fatty acid composition to evaluate the impact of disease stress on heart fatty acid composition.*

Significance 1: We have obtained viable samples of human myocardium from the left and right atria, and left and right ventricles in an effort to define the composition of the human heart in failing and healthy fresh heart tissue.

The composition of phospholipids, especially the sum of EPA and DHA in red blood cell (RBC) membranes (the Omega-3 Index) has been proposed as a clinical biomarker of cardiovascular health. With EPA+DHA values greater than 4.0% of total phospholipids as an indicator of heart health.

Aim 2: *To compare the fatty acid composition of human red blood cell and atria*

Significance 2: Dietary evidence exists in numerous animal studies and limited human studies of the direct effect of heart membrane incorporation of dietary eicosapentaenoic and docosahexaenoic acids. Little research of comparisons of RBC EPA+DHA to atrial membrane EPA+DHA in humans has been made, but the comparison of RBC EPA+DHA to left ventricular membrane EPA+DHA is yet to be determined.

1.7. RESEARCH QUESTIONS

Animal studies show fish oil fatty acids (EPA and/or DHA) in the diet are important, but analysis shows DHA is the major contributor to heart myocyte membranes and n-3 PUFA has been shown to benefit experimental hypertrophy and heart failure. Human epidemiological and diet intervention research show that the n-3 PUFA, EPA and DHA, may be important for optimal healthy heart function and a poor dietary intake of fish, hence poor in n-3 PUFA has been associated with an increased risk of cardiovascular disease, including heart failure and sudden cardiac death (Siscovick*, et al.*, 2000; Albert*, et al.*, 2002; Harris*, et al.*, 2004b).

What is the fatty acid composition of atrial and ventricular myocardial membranes, obtained from viable myocardium sourced from healthy and failing human hearts? Could heart failure be associated with low n-3 polyunsaturated fatty acids in the myocardial membrane? Are there differences between the left side of the heart and the right side of the heart in consideration of the different workloads and stresses? Atrial samples, as a readily accepted practice, are frequently obtained for medical diagnoses, but do they reflect the composition of the ventricle?

Dietary incorporation of the long-chain n-3 polyunsaturated fatty acids can be readily obtained from RBC and recently, atrial biopsy samples. Are the recent values found in these studies likely to be a true reflection of left ventricular membrane long-chain n-3 polyunsaturated fatty acids? Can the Omega-3 Index be validated for the left ventricle?

1.8. HYPOTHESIS

This study will test the hypothesis that human failing myocardial fatty acid composition is different from the healthy heart. The difference may be adaptive to the condition or the consequence of a nutritional (dietary) basis of the disorder. Results are interpreted in light of controlled animal studies and a minimum of human study. It will further test the hypothesis that (readily obtainable) red blood samples provide a valid and accurate marker of (usually inaccessible) cardiac ventricular fatty acid composition.

1.9. THESIS OUTLINE

Chapter 1 presents a review of the literature supporting the basis for the detailed investigation of normal human heart and failed human heart membrane phospholipid fatty acids.

Chapter 2 will characterise atrial and ventricular samples from healthy human donor and explanted failing human myocardium, and will additionally assess the relationship between the atrium and ventricles in these same samples.

Chapter 3 will demonstrate the efficacy of the RBC EPA+DHA (the Omega-3 Index) and right atrial membrane EPA+DHA comparisons and help to establish the Omega-3 Index as an integral part of a clinicians' assessment of heart health in humans.

Chapter 4 will combine data from healthy human heart, identifying the correlation between membrane omega-3 fatty acids of red blood cells, atrium and the left ventricle as a basis for the final conclusions.

Chapter 2

FATTY ACID COMPOSITION OF HUMAN HEART: COMPARISON OF ATRIA AND VENTRICLE, AND HEALTHY DONOR AND EXPLANTED (FAILING) HEARTS

2.0. INTRODUCTION

The impetus for focusing much research on the influence of dietary fatty acids on the heart arose from the initial observations by Dyerberg and Bang during the 1970's of the low occurrence of ischaemic heart disease amongst Inuit populations of the northwest Greenland coast despite a diet high in cholesterol and animal fat (Dyerberg*, et al.*, 1975, 1989). The Inuit diet was rich in the long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), EPA (eicosapentaenoic acid, 20:5n-3[2]) and DHA (docosahexaenoic acid, 22:6n-3), obtained largely from marine sources (Bang*, et al.*, 1980; Abete*, et al.*, 1999). Dyerberg and Bangs' observations directed much of the subsequent research interest towards the prevention of cardiovascular diseases (CVD) from thrombus formation and atherosclerosis (Dyerberg*, et al.*, 1978a; Dyerberg*, et al.*, 1978b). However, the Inuit diet resulted in plasma cholesterol, triglyceride and lipoprotein fractions that were much lower than habitual diets of Danes or, Inuit consuming a Danish diet.

The original diet-heart hypothesis that a high intake of dietary saturated fat induces increases in serum cholesterol which leads to atherosclerosis and coronary heart disease deaths (Keys, 1956; Keys*, et al.*, 1957) has remained controversial for many years (Bernard*, et al.*, 1999; Taubes, 2001; Steinberg, 2004). A new diet-heart paradigm (Siscovick*, et al.*, 2003) focuses instead on the closer associations of dietary n-3 PUFA to beneficial effects in the heart which has been supported by results from trials in post-myocardial infarction patients (Burr*, et al.*, 1989; Valagussa*, et al.*, 1999; Marchioli*, et al.*, 2002). However, the epidemiology linking fish consumption to prevention of fatal arrhythmias (Gloster*, et al.*, 1969; Bang*, et al.*, 1972; Marchioli*, et al.*, 2002), heart

[2] n-, is the current IUPAC notation used to describe the location of the first double bond in the carbon chain from the terminal or omega (ω) end of the particular fatty acid of interest. Fatty acids are designated by chain length: number of double bonds.

failure (Albert, *et al.*, 2003; Mozaffarian, *et al.*, 2005a; Tavazzi, *et al.*, 2008a) and lowering of the heart rate (Besse, *et al.*, 1994; Mori, *et al.*, 1999; Mozaffarian, *et al.*, 2005b; O'Keefe, *et al.*, 2006) suggests mechanisms associated with much lower intakes and for reduced cardiovascular mortality other than those classically associated with ischaemic heart disease. Animal experimental studies have consistently demonstrated a link between myocardial membrane fatty acid composition and heart function (Pepe, *et al.*, 1996, 2002; McLennan, *et al.*, 2007; Abdukeyum, *et al.*, 2008) underpinning fatal arrhythmias (McLennan, 2004) and heart failure (McLennan, *et al.*, 2012).

Docosahexaenoic acid is an important and unusual fatty acid in that it has the greatest number of double bonds (six within its twenty-two carbon chain) found in mammalian cell membranes. These double bonds provide a flexibility unmatched by other fatty acids which disrupts cholesterol molecules from lateral packing within the cell membrane bilayer construction and their protein interactions (al Makdessi, *et al.*, 1995). The other major n-3 PUFA EPA has been recognised for its potential role as a substitute for AA in a variety of enzymatic and non-enzymatic pathways relating to bioactive eicosanoids. More recently, DHA has been identified as a substrate for many of these pathways leading to other bioactives (resolvins, protectins) that might also contribute to the effects of fish oil (Serhan, *et al.*, 2002). This fatty acid is found in large concentrations in the membranes of specific vital organs, such as the brain and retina (Arterburn, *et al.*, 2006) and the hearts of several animals species. As yet, the membrane phospholipid fatty acids of the myocardium of humans are yet to be fully described. With DHA potentially to be the bioactive constituent in bestowing cardioprotection against ischaemic arrhythmias (McLennan, *et al.*, 1996), the membrane phospholipid fatty acid composition of human cardiomyocytes is generalized from feeding studies in animals.

Heart membrane phospholipid fatty acid composition has been well characterised in many animals; (with some examples given in Table 2.1) for rat (Gudbjarnason, *et al.*, 1978c; Charnock, *et al.*, 1983; Reibel, *et al.*, 1986); marmoset (non-human primate)(Charnock, *et al.*, 1983); guinea pig (Abedin, *et al.*, 1999); mouse (Huggins, *et al.*, 2009); pig (Nair, *et al.*, 1999); dog (van der Vusse, *et al.*, 1982; Billman, *et al.*, 2010); and cat (Reibel, *et al.*, 1986). All animals show altered cell membrane composition in response to variation in dietary fatty acids. It is therefore often difficult to know what the baseline is, as base diets will typically vary from laboratory to laboratory. However, it is recognized that some basic differences arise between species that can be characterised according to body surface area and metabolic rate. A major finding is that the long-chain n-3 PUFA DHA is present in high concentrations in myocardial membranes as animal body size decreases and metabolic rate increases. From this observational comparative physiology it has been proposed that increased DHA incorporation induces increased metabolic rate and increased heart rate (Bertrand, *et al.*, 2008). However, there is overwhelming evidence from both animal dietary studies and human epidemiology, individual clinical trials and meta-analysis of clinical trials with heart rate as secondary outcomes, that increasing dietary intake of DHA (which in animals is clearly associated with increased myocardial DHA) actually slows the heart rate (Mori, *et al.*, 1999; Mozaffarian, *et al.*, 2005b; Mozaffarian, *et al.*, 2006b; O'Keefe, *et al.*, 2006; McLennan, *et al.*, 2012). It is therefore suggested that rather than causing increases in heart rate, myocardial DHA increases as an adaptive response to increased metabolic rate (McLennan, *et al.*, 2005). Furthermore, there is evidence of adaptive increases in membrane DHA in animal models of cardiovascular stress including pressure overload hypertrophy (Reibel, *et al.*, 1986) and catecholamine stress (Gudbjarnason, *et al.*, 1975b; Benediktsdottir, *et al.*, 1988). Adaptive increases in DHA

are also recorded in skeletal muscle of man and rats, in response to exercise stress independent of diet (Helge*, et al.*, 1999; Helge*, et al.*, 2001). This brings us to the question of adaptive and/or dietary changes in the human heart.

While most data from animals comes from ventricular sections obtained fresh under laboratory controlled sampling and storage conditions, much of the human data (Table 2.2) is obtained from samples taken from cadavers with varying causes of death (including cardiac) and time delays after death (Fletcher, 1972; Gudbjarnason*, et al.*, 1975a; Belfrage*, et al.*, 1979; Sexton*, et al.*, 1995). Alternatively, most fresh tissue for analysis is atrial (Harris*, et al.*, 2004a; Garg*, et al.*, 2006; Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010) or papillary muscle biopsy (Rocquelin*, et al.*, 1985; Rocquelin*, et al.*, 1989) taken as a secondary outcome during surgery, or ventricular endothelial biopsy taken to monitor rejection in transplanted hearts (Harris*, et al.*, 2004a). Great variation is seen across the n-3 and n-6 PUFA of interest from these previous investigations and limited to, when identified, a single heart chamber. It is unknown whether the variations are related to the sample location (chamber) or collection conditions. Therefore translation of animal data to clinical application is limited by the incomplete characterization of human heart and lack of direct comparison of tissues.

In order to fill the gap in the knowledge base of human heart membrane phospholipid fatty acid composition, this study evaluated the membrane fatty acid composition of all four cardiac chambers within each heart using fresh myocardial samples. Samples were freshly obtained from explanted failing hearts and from viable healthy donor hearts made available through a heart transplant program.

Table 2.1. Heart Membrane Phospholipid Fatty Acid Composition from Differing Animal Species Consuming Control Diets.

			Selected Myocardial Membrane Phospholipid Fatty Acids					
			n-6 PUFA		n-3 PUFA			
Author (year)	**Sample Type**	**n**	**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**
Gudbjarnason, et al., (1978)	Rat Heart	12	26.7	15.4	*n.d.*	0.2	1.5	12.4
Van der Vusse et al., (1982)	Dog Left Ventricle	5	25.2	28.3	*n.r.*	0.4	*n.r.*	0.5
Charnock, et al., (1983)	Rat Atria	13	13.5	21.1	*n.d.*	*n.d.*	1.3	8.3
	Rat Ventricle	11	24.9	15.9	*n.d.*	*n.d.*	1.0	12.3
Charnock, et al., (1985)	Marmoset Atria	6	15.0	18.0	*n.d.*	*n.r.*	2.3	3.5
	Marmoset Ventricle	6	21.8	16.9	0.1	*n.r.*	2.0	3.4
Reibel et al., (1986)	Cat Right Ventricle	5	18.8	14.8	*n.r.*	*n.r.*	1.8	14.4
Abedin,et al., (1999)	Guinea Pig	12	24.6	23.9	*n.r.*	*n.r.*	0.8	0.3
Nair et al., (1999)	Pig	7	6.1	3.1	0.1	0.2	0.3	0.3
Huggins et al., (2009)	Mouse Ventricle	3-5	16.3	10.6	*n.r.*	*n.d.*	*n.r.*	24.2
Billman et al., (2010)	Dog Right Atria	6	*n.r.*	*n.r.*	*n.r.*	0.4	*n.r.*	0.6

AA - arachidonic acid, 20:4n-6 ; ALA - alpha-linolenic acid, 18:3n-3; LA - linoleic acid, 18:2n-6; EPA - eicosapentaenoic acid, 20:5n-3; DPA - docosapentaenoic acid, 22:5n-3; DHA - docosahexaenoic acid, 22:6n-3. *n.d.* - not detected; *n.r.* - not recorded. n = number per group.

Table 2.2. A Review of Relevant Past Human Heart Membrane Studies

Author (year)	Sample Type	n	Selected Myocardial Membrane Phospholipid Fatty Acid (means±SD % of total)						Comments
			n-6 PUFA		n-3 PUFA				
			LA	AA	ALA	EPA	DPA	DHA	
Gudbjarnason and Hallgrimsson (1975, 76)	Cadaveric	2	*n.r.*[1]	17.8	*n.r.*	*n.r.*	*n.r.*	*n.r.*	AA/DHA ratio declines with age; Limited fatty acids reported; small sample numbers
Gudbjarnason *et al.* (1978)	Cadaveric	151	17.6±0.7	17.9±0.5	*n.r.*	*n.r.*	*n.r.*	5.6±0.3	Author note: DHA important in maintenance of normal heart rate; DHA increases in with age, LA decreases with age
Belfrage *et al*. (1979)	Cadaveric Right Ventricle	1 2	25.8 21.0	16.2 23.8	*n.r.* *n.r.*	*n.r.* *n.r*	*n.r.* *n.r.*	8.5 10.4	Heart numbers are small; sex unknown. Multiple samples taken from a single heart 15 hours post-mortem.
Shenolikar (1980)	Cadaveric	50	9.9	*n.r.*	*n.r.*	*n.r.*	*n.r.*	*n.r.*	Tissue samples were fixed in formalin
Rocquelin *et al*. (1985)	Left Papillary Muscle	M=29 F=24	18.9±0.4 19.4±0.6	23.4±0.4 22.5±0.4	0.2±0.02 0.2±0.02	0.5±0.08 0.5±0.08	1.6±0.04 1.5±0.06	5.1±0.2 5.3±0.2	Chronic rheumatic heart disease; Male age range 41-70 yr, Females age range: 0-80 yr
Sexton *et al*. (1995)	Cadaveric	M=54 F=25	16.8±3.7 18.6±3.9	15.7±4.6 13.9±3.7	0.2±0.1 0.2±0.1	0.5±0.4 0.3±0.2	1.5±0.5 1.1±0.4	3.3±1.0 2.8±1.3	Hearts from cardiac and non-cardiac deaths; Combined sex age range: 7-92 yr
Harris *et al*. (2004)	Left Interventricular Septum	21	9.1±2.8	9.1±3.6	0.3±0.2	0.2±0.1	0.8±0.5	1.5±0.8	Routine biopsy post-heart transplant; Age average 56 yr; Subject sexes Males=16, Females=5
Garg *et al*. (2006)	Right Atrial Appendage	9	16.9±0.8	21.9±1.8	0.1±0.1	0.8±0.3	2.1±0.15	6.7±1.2	CABG surgery; Age mean 60 yr; Males=8; Control subjects.
Metcalf *et al*. (2007)	Right Atrial Appendage	10	14.7±1.7	20.8±2.0	0.1±0.03	0.5±0.1	2.0±0.4	4.8±0.6	CABG surgery &/or valve replacement; Age 60.7±14.5 (mean±SD yr), Males=9
Metcalf *et al*. (2010)	Right Atrial Appendage	61	14.5±1.5	19.9±1.8	0.2±0.1	0.7±0.3	1.9±0.4	5.6±1.4	CABG surgery &/or valve replacement; Subjects from previous trial used here also

[1] *n.r.* - not reported; AA/DHA - Arachidonic acid/Docosahexaenoic acid; CABG - Coronary artery-bypass grafting; LA - Linoleic acid.

2.1. METHODS

2.1.1. SUBJECTS

The donor hearts available for transplantation came from individuals from the general community and were included in this study due to the unavailability of cross-matched recipients or the length of time for transplantation was exceeded, whilst the heart remained hypothermic in the cardioplegic and preservation solution. The explanted hearts (failing hearts) came from the patients on the heart transplant list. See Appendix for full phospholipid fatty acid composition; donor and explanted (failing) hearts.

There were no differences found between ages of human donor heart group (Table 2.3) (50.8±19.1 years) (means±SD) and human explanted heart group (Table 2.4) (48.8±12.7 years), p=0.8646. Further results for individual ages will be as part of grouped data; ages grouped into decades with exceptions as noted.

2.1.2. SAMPLES

Complete chamber sets comprising atria (left and right) and ventricle (left and right) were obtained from explant hearts, n=25 and from donor hearts, n=18. The age and gender distribution of donor and explant hearts are provided in Tables 2.3 and 2.4. All heart samples were obtained through Dr Salvatore Pepe, Laboratory Head of the Cardiac Surgical Research Laboratory, Alfred Hospital and the Baker Heart Research Institute, Melbourne. Explanted failing heart tissue was used with the approval of the Alfred Hospital Human Ethics Committee for Discarded Tissue Research. The non-failing donor hearts were made available when excluded from transplantation and

were approved for use in research by donor family consent and the Victorian Organ Donation Service, Australian Red Cross (Canton*, et al.*, 2011).

Table 2.3. Human Donor Hearts Gender and Age groups.

Ages[1]		**Gender**	
(years)	***N***	***Female***	***Male***
10 - 29	3	0	3
30 - 49	2	1	1
50 - 59	5	2	3
60 - 69	4	1	3
70 - 79	2	0	2
Total	**16**	**4 (25%)**	**12 (75%)**

[1] Heart ages grouped into decades except 10-29 years and 30-49 years.

Table 2.4. Human Explant (Failing) Hearts Gender and Age groups.

Ages[1]		**Gender**	
(years)	***N***	***Female***	***Male***
10 - 29	2	0	2
30 - 49	8	1	7
50 - 59	9	1	8
60 - 69	6	1	2
70 - 79	0	0	0
Total	**22**	**3 (14%)**	**19 (86%)**

[1] Heart ages grouped into decades except 10-29 years and 30-49 years.

2.1.3. SAMPLE PREPARATION AND FATTY ACID ANALYSIS

2.1.3.1. Human Heart Sample Preparation

i) Lipid Extraction

Ventricle and atria samples were stored at -80°C until ready for analysis. A modified Folch (Folch*, et al.*, 1956) lipid extraction technique was employed. The atrial tissue samples cleaned of obvious fatty material (20–50 mg wet weight) were homogenized

by hand, using all-glass Ten Broeck homogenisers (Pyrex™ or Wheaton™) in 4 mL of analytical grade chloroform:methanol (2:1, v/v); once homogenized the samples were left to rotate overnight in a cool room at 3°C. To all solvents 0.01% (w/v) butylated hydroxytoluene (BHT) was added to act as an antioxidant.

Following rotation, 2 mL of 1M H_2SO_4 was added and the sample tubes were shaken vigorously for 60 seconds, then placed in a Hettich Rotina 38R benchtop centrifuge and spun at 400*g* for 10 minutes. The top aqueous layer was removed and the bottom non-aqueous layer was conserved to clean glass screw-top test-tubes, the residual had a further 2 mL of 1M H_2SO_4 added, it was then shaken and centrifuged again for a further 10 minutes. The process of conserving the non-aqueous layer was repeated and added to the initial sample screw-top test-tubes.

To absorb any residual H_2O from the isolated lipid samples, a small amount of anhydrous sodium dithionite is added, after which the sample is filtered through silane treated glass wool. The purified lipid samples were placed in a heat block (average temperature of 25°C) and dried under a stream of nitrogen gas. Once dried the samples were removed from heat block, reconstituted in 5 mL of hexane and placed in the freezer ready for phospholipid separation.

ii) Phospholipid Fraction Isolation

To isolate the phospholipid fatty acid fraction, the samples were applied to silica solid phase extraction (SPE) cartridges (Certified Sep-Pak™ SPE). Chloroform, 3 x 10 mL, was used to elute cholesterol esters and free fatty acids, then 3 x 10 mL chloroform: methanol (4:1, v/v) was used to elute the triacylglycerides, leaving the remaining phospholipids to be eluted from the silica cartridge with 3 x 10 mL methanol wash.

iii) Fatty Acid Methyl Ester (Fame) Formation

The phospholipid fatty acids were methylated to fatty acid methyl esters (FAME) in preparation for gas chromatography. The samples were collected into clean screw-top tubes, placed in a heat block (average temperature of 25 °C) and dried down under nitrogen gas to pure lipid, to which 2 mL methanol: toluene (4:1, v\v) was added. In a fumehood, whilst vortexing, 200 µL acetyl chloride was added, the samples where then placed in the heat block for 60 minutes at 100 °C. At the end of 60 minutes the samples were quickly removed from the heat block and rapidly cooled in ice, to then be centrifuged at 4000 *g* for 10 minutes. The resultant lipid layer was then pipette into clean deactivated borosilica glass vials, using 250 µL glass inserts.

iv) Gas Chromatography and Quantification of Fatty Acid Methyl Esters

The fatty acid methyl esters were analysed by flame ionizing detector - gas chromatography (FID-GC) using a Shimadzu GC-17A chromatograph with the automated sample injection system, AOC-20i. The capillary column was a Varian CP-Select™ CB for FAME (Middleburg, Netherlands; catalogue number: CP7419) at 50 m x 0.25 µm x 0.25 mm (length x wall coating x internal diameter). For each sample run, consisting of 10 to 25 samples, two FAME reference standards were used (NuChek Prep, Elysian, MN, USA; catalogue number: GLC 673B and Sigma-Aldrich Qualitative F.A.M.E. Mix, C4-C24; catalogue number: 18919-1AMP) (Figure 2.1). A 1µL sample was injected into the split system using hydrogen gas as the carrier, with a temperature ramp function commencing at 170°C and rising to 210°C over 24 minutes. Ionizing detector results are graphically depicted as peaks utilising the Shimadzu Chromatopac Class-VP™ Version 7.2.1 SP1 software.

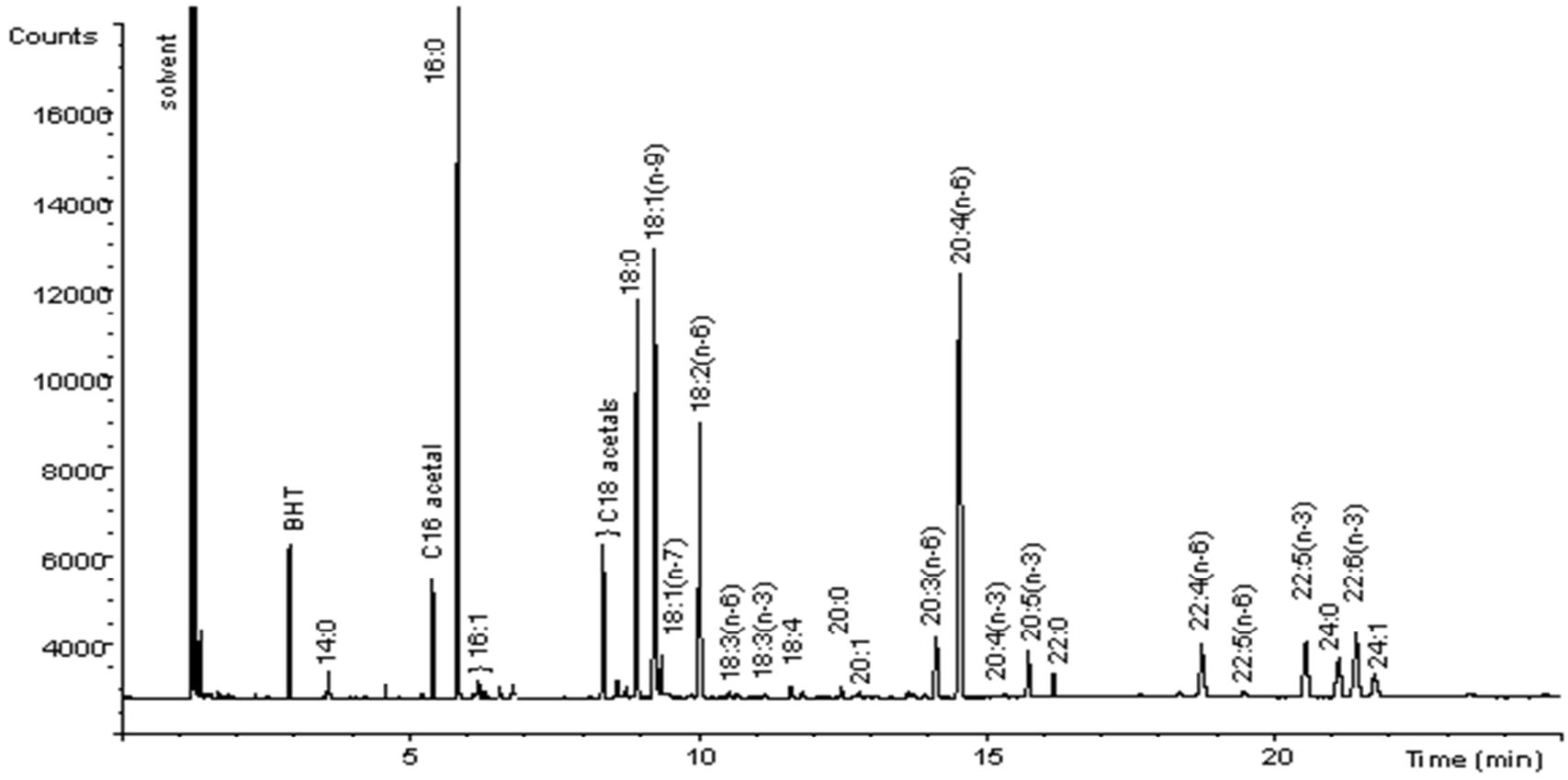

Figure 2.1. Typical chromatogram from FID-GC of the Sigma-Aldrich Qualitative FAME standard (C4-C24). Peaks are identified on the FAME standard as shown here labelled and are used to identify sample analyte fatty acids. Signal strength on Y-axis and time in minutes on X-axis.

2.1.4. CHEMICALS

All chemicals, solvents and laboratory consumables are of analytical grade supplied by Crown Scientific Pty Ltd (Ebos Group Ltd., Minto, NSW), Sigma-Aldrich Corporation (Castle Hill, NSW), Phenomenex Australia Pty Ltd (Pennant Hills, NSW), BOC Gases Australia Ltd (North Ryde, NSW), Waters Corporation Australia (Rydalmere, NSW), Wheaton Science Products (Wheaton Industries Inc., Millville, NJ, USA) and NuChek Prep Inc. (Elysian, MN, USA).

2.1.5. STATISTICAL ANALYSIS

Data was analysed using Statistix 9 Analytical Software (Tallahassee, FL, USA) and graphed using GraphPad Prism 5, Version 5.02 (GraphPad Software Inc., USA). Statistical tests include summary descriptive two-sample T-test and paired-T-tests, and general analysis of variance (ANOVA) with fixed factors and post hoc multiple comparisons. When appropriate, linear regression and correlation analyses were conducted on individual fatty acids. Significance was accepted at $p<0.05$. Descriptive statistics are displayed as means and standard deviation (means±SD) or means and standard error of means (means±SEM).

2.2. RESULTS

2.2.1. DONOR HEARTS

2.2.1.1. Atria

Donor heart atrial phospholipid fatty acids comprised mainly of PUFA (49%) (Table 2.5) with 34% SFA and 16% total MUFA (Figure 2.2). At 40% the n-6 PUFA were the main PUFA with 21% AA and 17% LA compared to 6% DHA, which was the main n-3

PUFA (Figure 2.3), far exceeding the concentration of EPA (0.7%) and DPA (2%). At 0.1% or less, ALA was often undetectable.

When comparing left and right atria (Table 2.5) there was a small but statistically significant difference in the saturated fatty acid palmitic acid but no other differences were evident.

2.2.1.2. Ventricles

Donor heart ventricular phospholipid fatty acids comprised mainly of PUFA (54%) (Table 2.5) with 32% SFA and 14% total MUFA (Figure 2.2). At 46% total n-6 PUFA were the main PUFA with 23% AA and 21% LA compared to 4% DHA which was the main n-3 PUFA (Figure 2.3), less than 2% for DPA, less than 1% EPA and 0.2% ALA (Table 2.5).

Donor ventricles showed only minor differences between right and left chamber walls, with left being lower in total SFA and higher in total PUFA (Figure 2.2). Within the PUFA, AA and total n-6 PUFA were at significantly higher concentrations in the left ventricle (Table 2.5, Figure 2.3).

2.2.1.3. Atria versus Ventricles

Small differences between atria and ventricles were seen as significantly lower total SFA and total MUFA and significantly increased total PUFA in the ventricles (Figure 2.2). In the ventricles, LA and AA were found at similar concentrations (~22%), with LA significantly greater than atrial LA (17%) (Table 2.5). Ventricle total n-6 PUFA was significantly greater than atria total n-6 PUFA (Figure 2.3). Significantly lower total n-3 PUFA (Figure 2.3), lower DHA and EPA+DHA were found in the ventricles compared to atria (Table 2.5).

Table 2.5. Major Membrane Phospholipid Fatty Acids from Donor Human Hearts.

DONOR	Left Atria	Right Atria	Left Ventricle	Right Ventricle
N=	16	16	16	16
Major Saturated Fatty Acids[1] (% of total)				
16:0 (PA)	17.53±1.42[a]	15.64±2.87[b]	14.46±0.60[b]	15.18±0.81[b]
18:0 (SA)	16.15±1.70	16.78±2.30	15.76±1.60	16.55±1.32
Total SFA	34.87±1.74[a]	33.70±2.01[a,b]	31.45±1.39[c]	33.02±1.17[b]
Major Monounsaturated				
16:1n-7 (PoA)	1.52±0.83[a,b]	2.48±1.20[a]	0.61±0.50[b]	2.05±1.81[a]
18:1n-9 (OA)	11.51±1.69[a]	10.78±1.89[a,b]	9.71±1.35[b]	10.10±1.59[a,b]
Total MUFA	16.33±2.22[a]	15.90±1.97[a]	13.11±1.76[b]	14.78±1.86[a,b]
Major n-6 Polyunsaturated				
18:2 (LA)	17.41±1.97[b]	16.67±1.34[b]	21.64±2.04[a]	21.58±1.86[a]
20:4 (AA)	21.54±1.62[b]	22.52±1.91[b]	24.44±2.13[a]	22.20±2.36[b]
Total n-6	40.68±2.37[c]	41.28±2.10[c]	47.97±1.68[a]	45.58±1.87[b]
Major n-3 Polyunsaturated				
18:3 (ALA)	0.13±0.07[b,c]	0.08±0.08[c]	0.21±0.07[a]	0.18±0.08[a,b]
20:5 (EPA)	0.63±0.31	0.74±0.30	0.75±0.39	0.61±0.32
22:5 (DPA)	1.98±0.45	2.15±0.41	1.94±0.43	1.75±0.46
22:6 (DHA)	5.17±1.51[a,b]	6.12±2.08[a]	4.42±1.23[b]	4.06±1.13[b]
Total n-3	7.97±1.71[a,b]	9.12±2.24[a]	7.38±1.51[b]	6.62±1.42[b]
Total PUFA	48.65±2.76[c]	50.40±2.86[b,c]	55.34±1.31[a]	52.20±2.15[b]
EPA+DHA	5.81±1.74[a,b]	6.86±2.33[a]	5.17±1.56[b]	4.67±1.38[b]
AA:DHA ratio	4.2	3.7	5.5	5.5
DHA:DPA	2.8	3.0	2.4	2.5

[1] AA, Arachidonic acid; ALA, a-linoleic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic acid. MUFA, Monounsaturated fatty acids; OA, Oleic acid; PA, Palmitic acid; PoA, Palmitoleic acid; PUFA, Polyunsaturated fatty acids; SA, Stearic acid; SFA, Saturated fatty acids. Results are means±SD. [a, b] and [c] superscripts denote significant differences, $p<0.05$, between donor heart chambers.

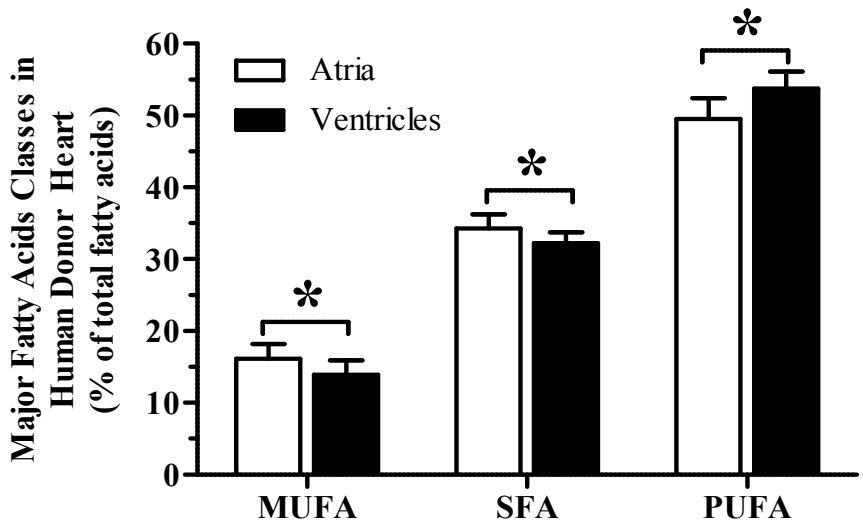

Figure 2.2. Atrium compared to ventricles for total MUFA, total SFA and total PUFA in Human Donor Hearts.

In human donor hearts differences were observed when upper and lower chambers were compared for total monounsaturated, total saturated and total polyunsaturated fatty acids. Donor atrium, open columns (□) and donor ventricles, filled columns (■). *means±SD are statistically different, $p<0.05$.

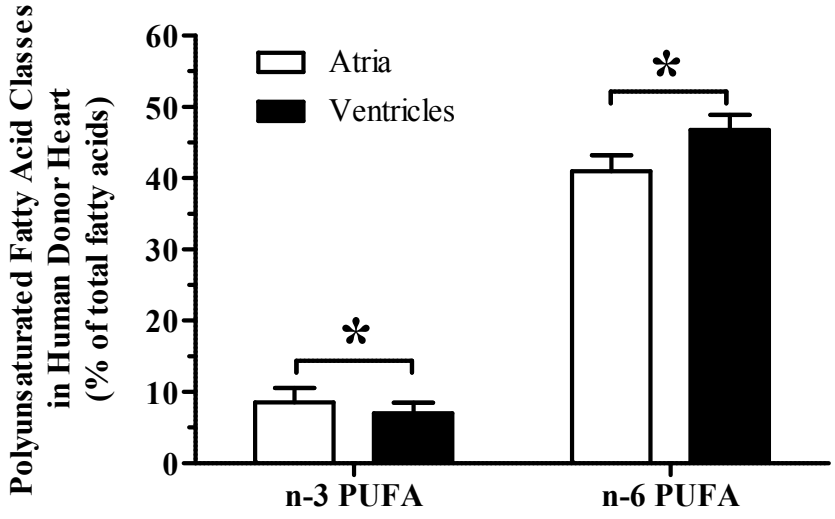

Figure 2.3. Atrium compared to ventricles for total n-3 PUFA and total n-6 PUFA in Human Donor Hearts.

In human donor hearts differences are observed when upper and lower chambers were compared for total n-3 PUFA and total n-6 PUFA. Donor atrium, open columns (□) and donor ventricles, filled columns (■).*means±SD are statistically different, $p<0.05$.

2.2.2. EXPLANT (FAILING) HEARTS

2.2.2.1. Atria

Explant (failing) heart atrial phospholipid fatty acids comprised mainly of PUFA (48%) (Table 2.6) with 35% SFA and 17% total MUFA (Figure 2.4). At 39% the n-6 PUFA were the main PUFA with 20% AA and 18% LA compared to 6% DHA, which was the main n-3 PUFA (Figure 2.5), far exceeding the concentration of EPA (0.7%) and DPA (1.7%). At 0.2% or less, ALA was often undetectable.

In the left compared to right atria (details in Table 2.6), there was a higher but statistically significant different concentration in total SFA and the saturated fatty acid palmitic acid, with significant differences seen in lower monounsaturated fatty acid palmitoleic acid and lower n-3 PUFA docosapentaenoic acid. No other significant differences were evident.

2.2.2.2. Ventricles

Explant heart ventricular phospholipid fatty acids comprised mainly of PUFA (51%) (Table 2.6) with 34% SFA and 15% total MUFA (Figure 2.4). At 43%, the total n-6 PUFA were the main PUFA with 21% AA and 20% LA, compared to 4.9% DHA which was the main n-3 PUFA (Figure 2.5), less than 2% for DPA, less than 1% EPA and 0.22% for ALA (Table 2.6).

Explant ventricles showed only minor differences between left and right chamber walls, with left being lower in total SFA and total MUFA and higher in total PUFA. Minor differences are seen between chambers but no significant differences were evident (Table 2.6).

2.2.2.3. Atria versus Ventricles

Small differences between atria and ventricles were seen as significantly lower total MUFA and significantly increased total PUFA in the ventricles (Table 2.6). In the ventricles, LA and AA were found at similar concentrations (~20%), with LA significantly greater than atria LA (18%) (Table 2.6). Ventricle total n-6 PUFA (43%) (Table 2.6) was found to be significantly greater than atria total n-6 PUFA (39%). It was found that total n-6 PUFA was significantly higher and total n-3 PUFA significantly lower in ventricle (Figure 2.5), including lower DHA and EPA+DHA (Table 2.6).

2.2.3. HUMAN DONOR HEARTS COMPARED TO EXPLANTED HUMAN HEARTS

2.2.3.1. Atria

There was a significantly lower relative concentration of total n-6 PUFA and total PUFA in explanted atria than in donor atria, whereas total MUFA was higher in explanted atria (p=0.0131) (Table 2.7). The individual n-6 PUFA arachidonic acid and n-3 PUFA DPA were significantly lower in explanted than donor atria (Table 2.7). The ratio of DHA/DPA in explanted atria (3.82±0.25) was significantly higher than in donor atria (2.87±0.21) (p=0.0047) (Figure 2.6).

2.2.3.2. Ventricles

There was a significantly lower relative concentration of n-6 PUFA linoleic acid and arachidonic acid, total n-6 PUFA and total PUFA in explanted ventricles than in donor ventricles (Table 2.7). There was significantly lower relative concentration of n-3 PUFA DPA in explanted ventricles but higher relative concentration of DHA (Table 2.7). The ratio of DHA/DPA in explanted ventricles (3.32±0.23) was significantly higher than in donor ventricles (2.44±0.16) (p= 0.0027) (Figure 2.7).

Table 2.6. Major Membrane Phospholipid Fatty Acids from Explanted (Failing) Human Hearts.

EXPLANTED	Left Atria	Right Atria	Left Ventricle	Right Ventricle
N=	22	22	22	22
Major Saturated Fatty Acids[1] (% of total)				
16:0 (PA)	18.00±1.21[a]	16.41±2.38[b]	15.58±1.42[b]	16.32±1.33[b]
18:0 (SA)	16.68±1.82	15.92±1.75	17.810±1.58	17.02±1.29
Total SFA	35.91±2.60[a]	33.75±2.11[b]	33.90±2.19[b]	34.71±2.13[a,b]
Major Monounsaturated				
16:1n7 (PoA)	1.41±1.15[b]	2.87±1.77[a]	1.25±0.80[b]	1.87±1.98[a,b]
18:1n9 (OA)	12.13±1.75[a,b]	12.18±1.54[a]	10.38±1.32[c]	10.91±1.58[b,c]
Total MUFA	16.86±1.89[a,b]	17.79±2.12[a]	14.20±1.96[c]	15.35±3.22[b,c]
Major n-6 Polyunsaturated				
18:2 (LA)	18.13±2.20[b]	17.26±2.11[b]	20.49±2.33[a]	20.23±2.45[a]
20:4 (AA)	19.25±2.45[b]	20.41±2.48[a,b]	21.70±3.10[a]	20.95±2.82[a,b]
Total n-6	39.14±2.83[b]	39.40±3.27[b]	44.32±3.49[a]	42.70±3.29[a]
Major n-3 Polyunsaturated				
18:3 (ALA)	0.20±0.08	0.19±0.10	0.22±0.09	0.22±0.07
20:5 (EPA)	0.64±0.37	0.73±0.29	0.67±0.29	0.68±0.27
22:5 (DPA)	1.53±0.43[b]	1.79±0.33[a]	1.63±0.38[a,b]	1.55±0.36[a,b]
22:6 (DHA)	5.47±1.80[a,b]	6.33±1.92[a]	4.97±1.59[a,b]	4.78±1.56[b]
Total n-3	7.87±1.94[a,b]	9.06±1.99[a]	7.53±1.63[b]	7.24±1.60[b]
Total PUFA	47.01±3.56[c]	48.46±3.19[b,c]	51.85±3.01[a]	49.94±3.87[a,b]
EPA+DHA	6.12±2.03[a,b]	7.06±2.11[a]	5.63±1.74[a,b]	5.46±1.71[b]
AA:DHA ratio	3.5	3.2	4.4	4.4
DHA:DPA	4.0	3.7	3.3	3.3

[1]AA, Arachidonic acid; ALA, α-linoleic acid; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic acid. MUFA, Monounsaturated fatty acids; OA, Oleic acid; PA, Palmitic acid; PoA, Palmitoleic acid; PUFA, Polyunsaturated fatty acids; SA, Stearic acid; SFA, Saturated fatty acids. Results are means±SD. [a, b] and [c] superscripts denote significant differences between explant heart chambers, $p<0.05$.

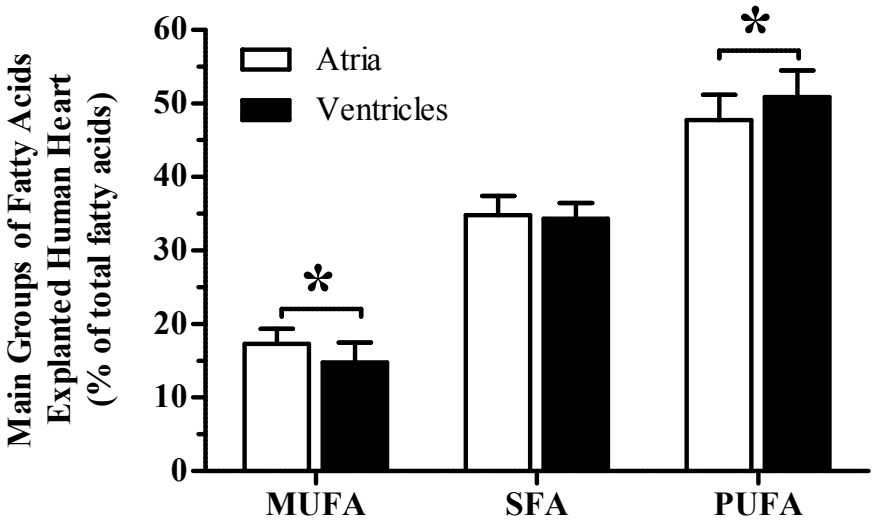

Figure 2.4. Atrium compared to ventricles for total MUFA, total SFA and total PUFA in Explanted Failing Human Hearts.

In explanted human hearts, differences are observed when upper and lower chambers are compared in total monounsaturated, total saturated and total polyunsaturated fatty acids. Explanted human atrium, open columns (□) and explanted human ventricles, filled columns (■). *means±SD are statistically different, $p<0.05$.

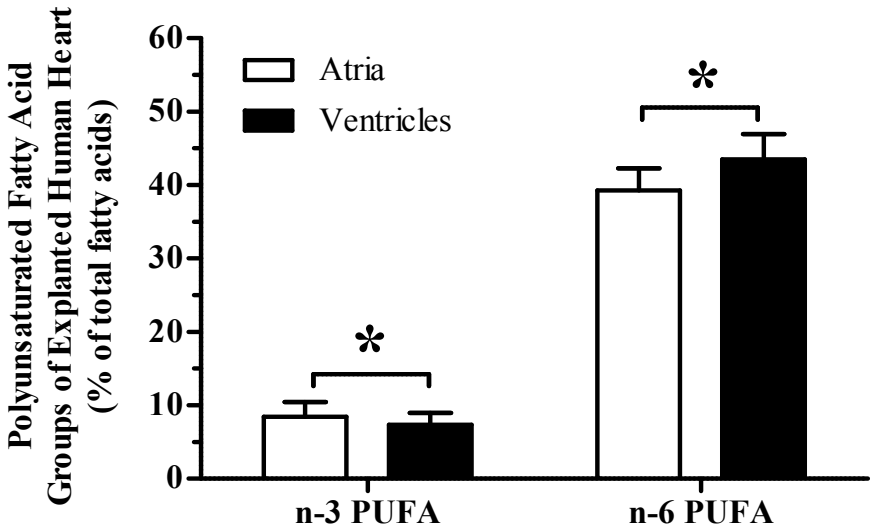

Figure 2.5. Atrium compared to ventricles for total n-3 PUFA and total n-6 PUFA in Explanted Failing Human Hearts.

In explanted human hearts, differences are observed when upper and lower chambers are compared for total n-6 PUFA and then total n-3 PUFA. Explanted human atrium, open columns (□) and explanted human ventricles, filled columns (■). *means±SD are statistically different $p<0.05$.

Table 2.7. Comparison of human explanted hearts to human donor hearts in selected membrane phospholipid fatty acids in atria and ventricles.

Human Myocardial Membrane Phospholipid Fatty Acid					
Fatty Acid	Heart Chamber	Donor N=16	Explanted N=22	Change[1]	Significant *p*-values
18:2 (LA)[2]	Atria	17.0±0.3	17.7±0.3	⇑	n.s.
	Ventricles	21.6±1.9[b]	20.4±2.4[a]	⇓	0.0162
20:4 (AA)	Atria	22.0±1.8[b]	19.8±2.5[a]	⇓	<0.0001
	Ventricles	23.3±2.5[b]	21.3±2.9[a]	⇓	0.0027
Total n-6	Atria	40.9±2.2[b]	39.3±3.0[a]	⇓	0.0059
	Ventricles	46.8±2.1[b]	43.5±3.4[a]	⇓	<0.0001
22:5 (DPA)	Atria	2.1±0.4[b]	1.7±0.4[a]	⇓	0.0010
	Ventricles	1.8±0.4[b]	1.6±0.4[a]	⇓	0.009
22:6 (DHA)	Atria	5.6±0.3	5.9±0.3	⇑	n.s.
	Ventricles	4.2±1.2[a]	4.9±1.6[b]	⇑	0.0464
Total PUFA	Atria	49.5±2.9[b]	47.7±3.4[a]	⇓	0.0059
	Ventricles	53.8±2.4[b]	50.9±3.6[a]	⇓	0.0001
Total MUFA	Atria	16.1±2.1[a]	17.3±2.0[b]	⇑	0.0131
	Ventricles	13.9±2.0	14.8±2.7	⇑	n.s.

Results are % of total means±SD. [1]Change: represents either greater than (⇑) or lesser (⇓) concentrations of fatty acids in explant hearts compared to donor hearts. [2]AA, arachidonic acid; ALA, α-linoleic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid. PUFA, polyunsaturated fatty acid. [a] and [b] identify significant differences between hearts for those chambers and fatty acid with related *p*-values.

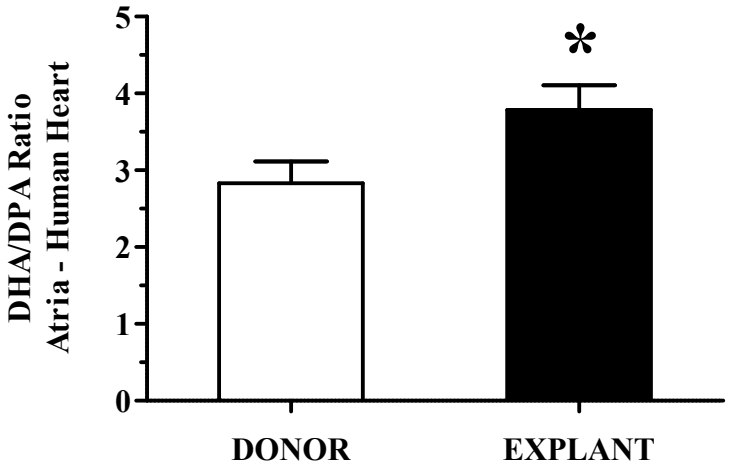

Figure 2.6. Mean DHA/DPA ratios found in atrium of donor and explanted human hearts.

Explanted atria have a significantly greater DHA/DPA ratio than is found donor heart atria (means±SEM), p=0.0047.

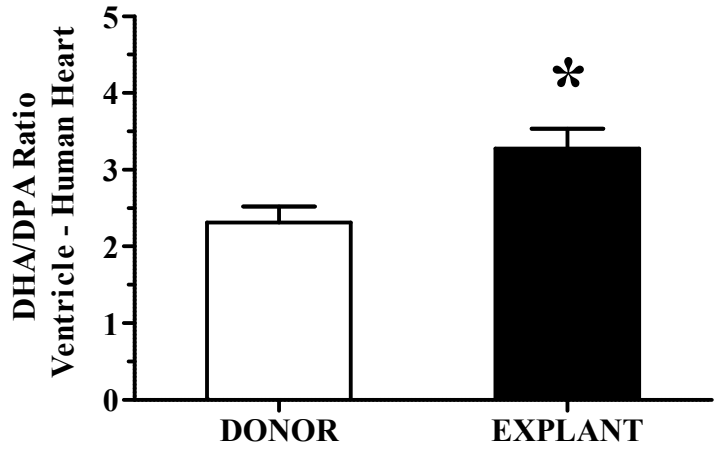

Figure 2.7. Mean DHA/DPA ratios found in ventricles of donor and explanted human hearts.

Explanted ventricles show a significantly greater DHA/DPA ratio than donor ventricle (means±SEM), p=0.0027.

2.2.4. FATTY ACID CLASSES FOUND IN ATRIUM AND VENTRICLES OF HUMAN HEART

The division of major fatty acid classes, SFA, MUFA and PUFA show similarities in percentage of total phospholipid fatty acids between atrium and ventricles in the human heart (Figure 2.8). The classes of n-6 and n-3 PUFA also follow a similar trend. The individual n-6 PUFA AA and n-3 PUFA DHA show clear differences in percentage of total phospholipid fatty acids between atria and ventricles (Figure 2.8).

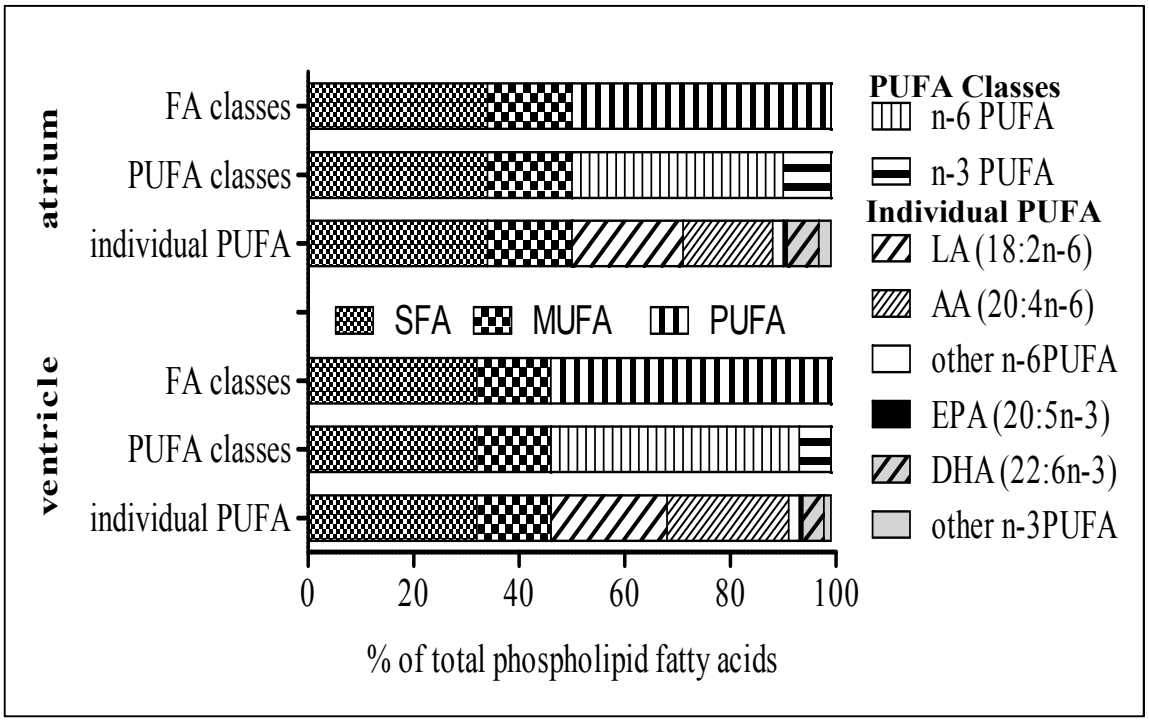

Figure 2.8. Division of major fatty acid classes and PUFA in the human heart.

Graphically the division of FA (fatty acid classes) and PUFA classes within atrium and ventricles tend towards similar trends, with individual fatty acids showing clear differences.

2.2.5. AGE AND THE HUMAN HEART

2.2.5.1. As Age Increases, DHA Increases in the Human Heart

There was a positive association between docosahexaenoic acid, the principle n-3 PUFA, and increasing age in all chambers of the human heart (combined donor and explant: LA, r^2=0.1123 and p=0.0199; RA, r^2=0.1697 and p=0.0033; LV, r^2=0.2140 and p=0.0003; RV, r^2=0.1627 and p=0.0023). There was no significant difference in slope of the regression line between donor and explanted human hearts (Figure 2.9).

2.2.5.2. As Age Increases, EPA+DHA Increases in the Human Heart

The n-3 PUFA, EPA+DHA, had a clear positive association with increasing age in all chambers of the human heart (LA, r^2=0.1550 and p=0.0068; RA, r^2=0.1725 and p=0.0041; LV, r^2=0.2305 and p=0.0003; RV, r^2=0.1645 and p=0.0023). The higher concentration of EPA+DHA was paralleled in both normal donor and failing explanted human hearts (Figure 2.10). The major contributor to EPA+DHA at all ages was DHA.

2.2.5.3. As Age Increases, the AA:DHA Ratio Decreases in the Human Heart

The ratio of AA/DHA reduced with increasing age in all chambers of the human heart (LA, r^2=0.2343 and p=0.0007; RA, r^2=0.1493 and p=0.0080; LV, r^2=0.2513 and p=0.0001; RV, r^2=0.1875 and p=0.0011) and was reflected in both normal donor and failing explanted hearts (Figure 2.11).

2.2.5.4. Age and the percentage of the Principal n-3 and n-6 PUFA

In donor hearts the n-3 PUFA, EPA and DHA (Table 2.8), were found to increase in the membrane phospholipid fatty acids over the human lifespan (Table 2.8). A statistically significant increase in membrane DHA in the 40's decade (p<0.0001, R^2 0.1931) from previous 10, 20 and 30's ages was seen. Concurrently, DPA was lower with increasing

age (Table 2.8). The main n-6 PUFA, LA or AA, show no clear age associations, apart from LA having a lower concentration in donor hearts during the 20's age decade. Arachidonic acid was statistically the least in the 30's age decade (Table 2.8).

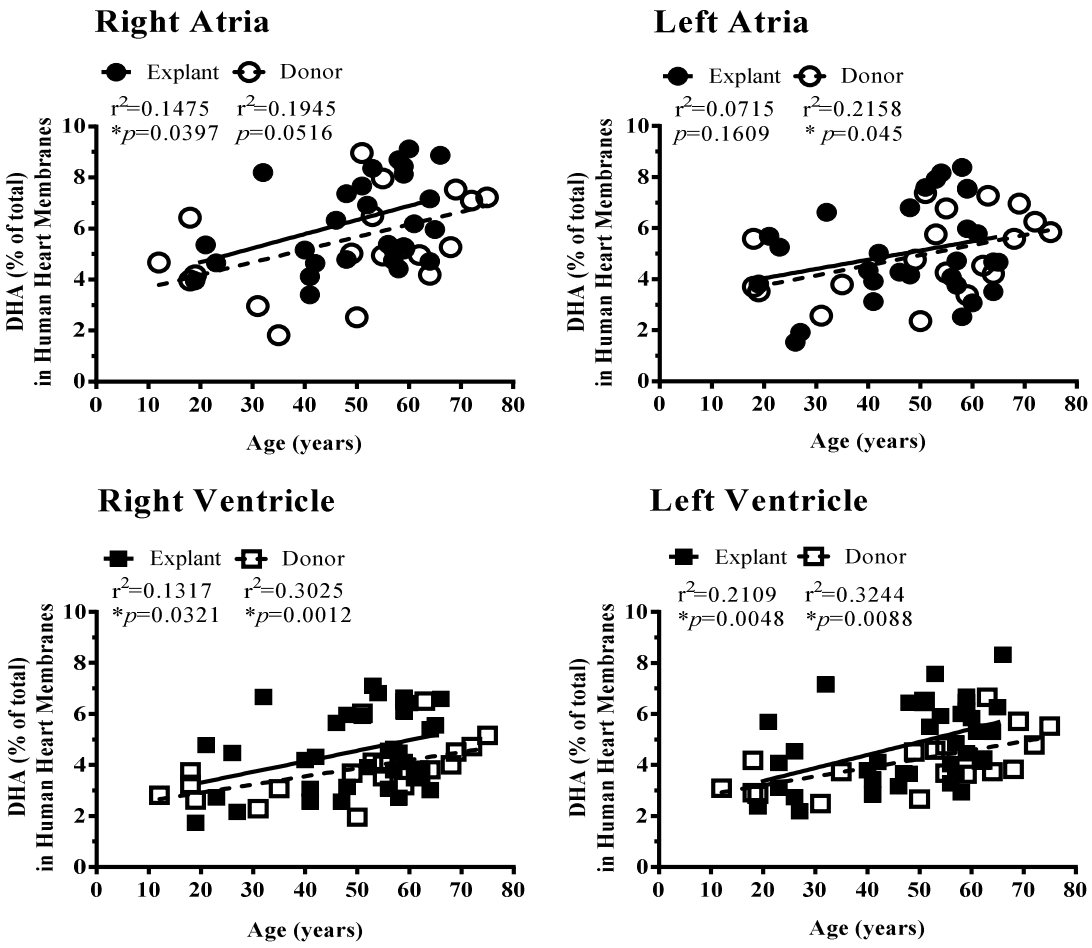

Figure 2.9. The association of age and human heart membrane phospholipid DHA.
In both normal donor and failing explanted hearts, DHA increases with age. Donor tissues are indicated by open symbols (○, □) and broken regression lines. Explanted tissues are indicated by filled symbols (●, ■) and solid regression lines. Atria are represented by circles and ventricles by squares. Goodness of fit for data indicated by r^2 value and slope of regression line significance indicated by *p*-value. *significant $p<0.05$.

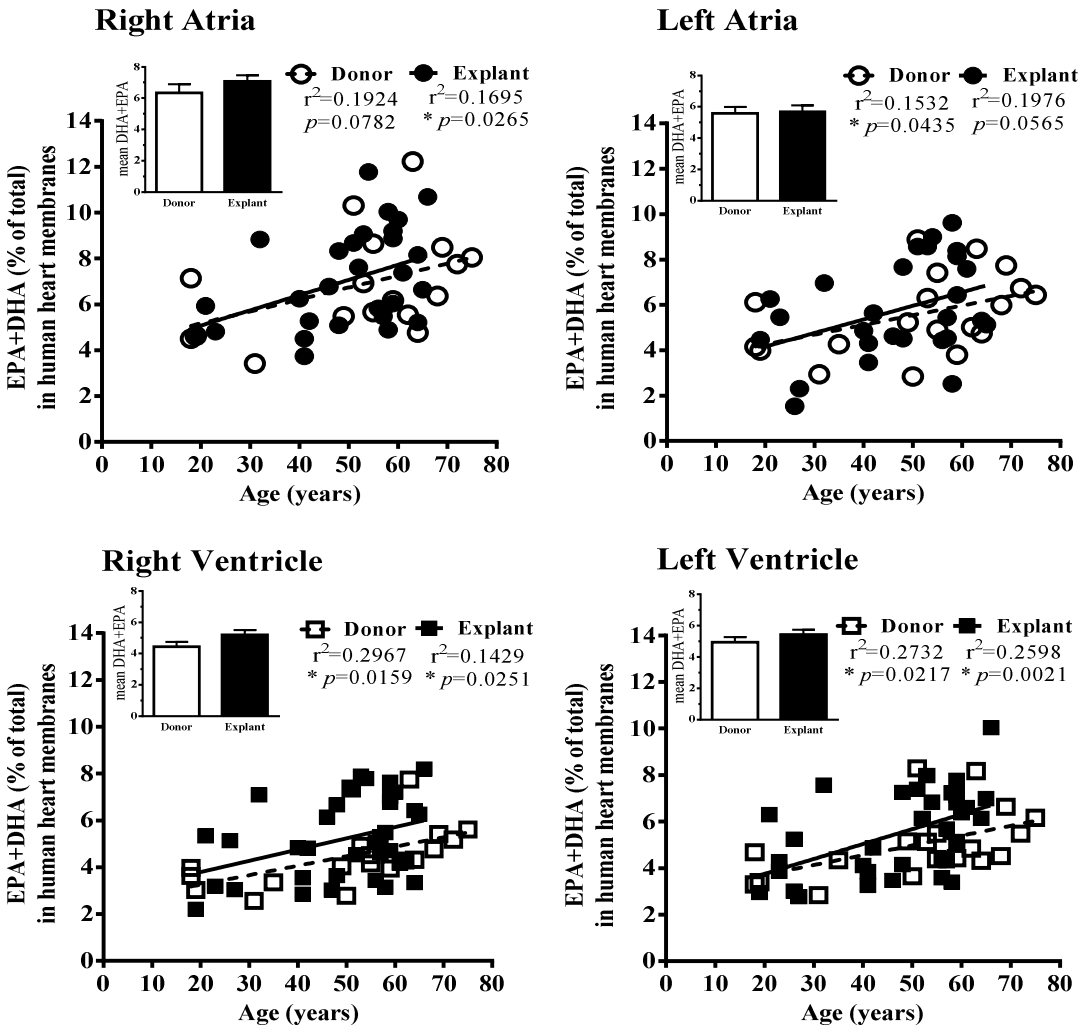

Figure 2.10. The association of age and EPA+DHA in human heart membranes.

In both normal donor and failing explanted hearts EPA+DHA increases with age. Donor atria are indicated by open circles (○) and for donor ventricles open squares (□), with dashed regression line. Explanted failing atria are indicated by filled circles (●) and explanted ventricles filled squares (■), with solid regression line. Goodness of fit for data indicated by r^2 value and slope of regression line significance indicated by *p*-value. *significant $p<0.05$.

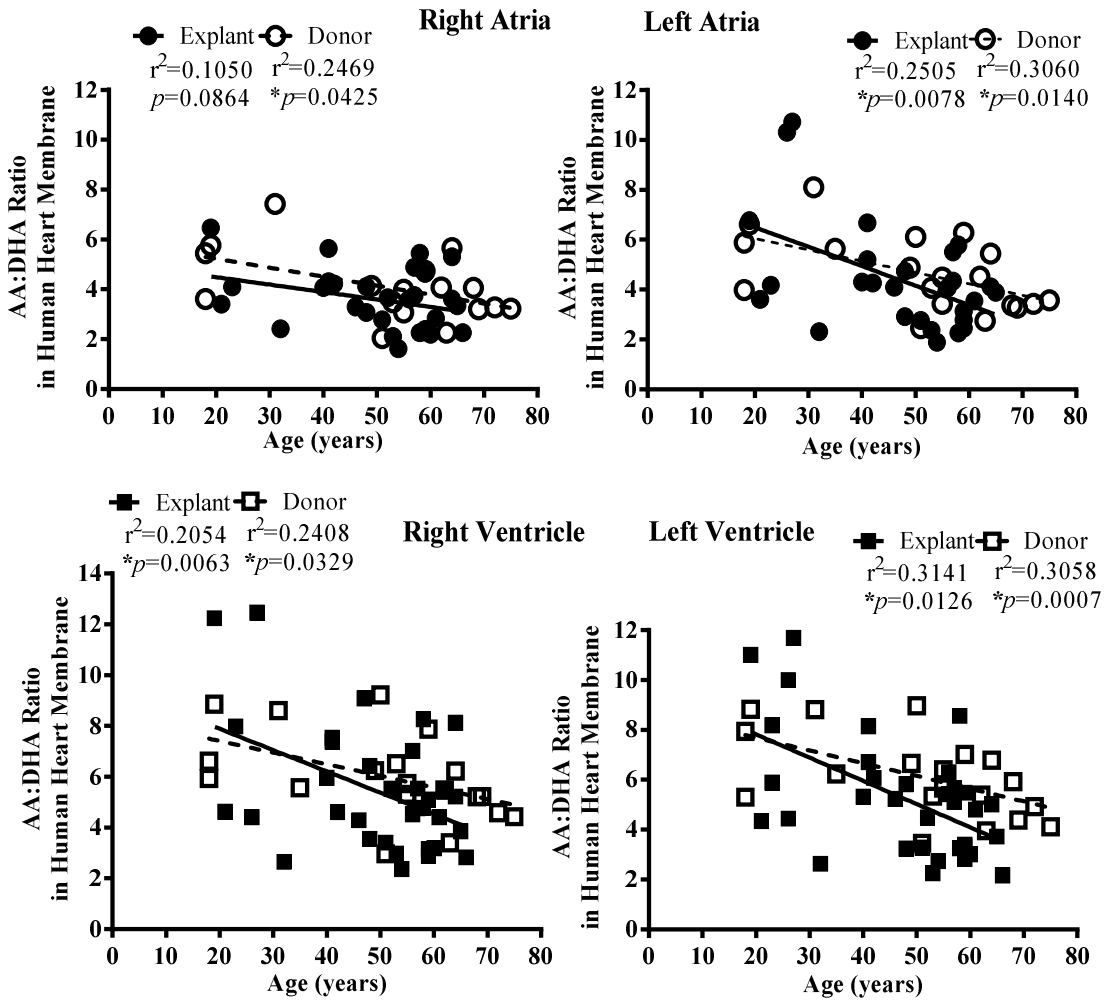

Figure 2.11. The association of age and human heart membrane AA:DHA ratio.

A declining AA:DHA ratio is observed in humans with age; a high AA:DHA ratio is purported to relate to a high risk of sudden cardiac death. Donor hearts are indicated by open circles for atrium (○) and open squares for ventricles (□) with dashed regression line. Explant hearts are indicated by filled circles for atrium (●) and filled squares for ventricles (■) with solid regression line. Goodness of fit for data indicated by r^2 value and slope of regression line significance indicated by p-value. * significant $p \leq 0.05$.

Table 2.8. Ageing in human donor hearts and the effects on the major n-3 PUFA and n-6 PUFA membrane phospholipid fatty acids.

Fatty Acid		Age[1]
n-6 PUFA	**LA**	No clear age association 20 < 60 < 10, 30, 40, 50 age groups
	AA	No clear age association 30 < all other ages*
n-3 PUFA	**EPA**	Increases with age up to 60 Increased significantly from 40*
	DPA	Decreases with age 10 > 20 – 60*
	DHA	Increases with age including 70's Increased significantly from 40**

[1] For ease of analysis, ages are grouped into decades, except the 60 year group, which also includes ≥70 year old.**10**: 10-19 years of age; **20**: 20-29 years of age; **30**: 30-39 years of age; **40**: 40-49 years of age; **50**: 50-59 years of age; **60**: 60-79 years of age. *p<0.05, **p<0.0001

2.2.6. GENDER DIFFERENCES IN THE HUMAN HEART

2.2.6.1. Donor Hearts

Seventy five percent of total donor hearts available for transplantation were from males (Table 2.3). Total PUFA in left atrium was found to be significantly greater in male hearts than female hearts, p=0.0406. There were trends towards significance in the right atrium with males greater than females in total n-3 PUFA, EPA and the n-6 PUFA, AA. In the left ventricle, total PUFA, n-3 PUFA and DPA were found to be greater in males than females.

2.2.6.2. Explanted (Failing) Hearts

Males were 86% of all subjects (Table 2.4). Left atrium EPA was greater in females than male and trended towards significance.

2.2.6.3. Female Hearts

The number of female explanted hearts (n=3) (Table 2.4) were similar in number to that of female donor hearts (n=4) (Table 2.3). In the left ventricle of donor hearts total n-6 PUFA was significantly greater than explant hearts, p=0.0043. The concentration of EPA was higher in explanted right atrium than in donors, p=0.312.

2.3. DISCUSSION

This study has enabled a complete characterisation of the phospholipid fatty acid composition of freshly obtained human heart across all four pumping chambers. This has revealed some subtle differences, yet an overall consistency between the different chambers of the human heart. These subtle differences may in part explain the differences in previous reports that come from different sampling sites within the heart and different collection and preservation conditions. Past reports generally fall into one of two groups. The first relied on tissue sampling from human cadavers (Fletcher, 1972; Gudbjarnason*, et al.*, 1975a; Belfrage*, et al.*, 1979; Sexton*, et al.*, 1995) at indeterminate times after death and without collection and preservation procedures associated with fresh tissue handling. The second group were able to freshly obtain small, intra-operative biopsy samples (Rocquelin*, et al.*, 1985; Rocquelin*, et al.*, 1989; Harris*, et al.*, 2004a; Garg*, et al.*, 2006; Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010) and acutely preserve them under optimal conditions. An assessment of the left ventricular fatty acid composition of explanted hearts found in this study compared most favourably to the

intra-operative left papillary muscle samples of Rocquelin *et al*. (1985), with similarities in the major n-6 PUFA (LA and AA) and n-3 PUFA (ALA, EPA, DPA and DHA) concentrations. The viable donor left ventricles analysed here are distinguished by greater LA and AA concentrations when compared to Rocquelin *et al*. (1985) samples. In turn, the intra-operative right atrial appendage samples of Garg *et al*. (2006) and Metcalfe *et al*. (2007, 2010) are largely in concordance with data presented here for right atrial samples. Therefore, these findings establish that intra-operative atrial or papillary muscle samples taken under the ethical constraints of minimal biopsy size during elective surgical procedures well-represent the true heart composition for those heart regions as confirmed in this study using the substantial tissue samples taken under conditions more akin to carefully controlled animal studies.

Membrane phospholipid fatty acid compositions are true for those sites sampled, with differences well described between tissues of origin. Data derived from right interventricular septum biopsies (Harris, *et al*., 2004a) stand in contrast to any cardiac tissue of this study or of the intra-operative atrial or papillary muscle biopsies previously reported, having much lower PUFA concentrations (both n-3 PUFA, and n-6 PUFA). Within that study, the low PUFA concentrations, including low DHA concentrations, are much more in line with cheek cell samples than with red blood cells or plasma phospholipid from the same subjects. The contrast to other ventricular samples and similarity to cheek cells suggests that these very small samples taken to assess immune function and tissue rejection may in fact be more ventricular epithelium than myocardium. Contrasting membrane composition is well described between tissue types within species. For example, in a non-human primate the marmoset monkey, markedly more DHA is recorded in excitable (neural, myocardial or skeletal muscle) tissue (Charnock*, et al.*, 1989; Charnock*, et al.*, 1992). In human tissue, DHA and other

PUFA vary between tissues (Lund, *et al.*, 1999; Baur, *et al.*, 2000; Connor, *et al.*, 2000; Harris, *et al.*, 2004a; Arterburn, *et al.*, 2006) although the differences reported between excitable and non-excitable tissue are not so clear cut and may be influenced by sampling procedures, (Arterburn, *et al.*, 2006) report heart composition from the human intraventricular septum biopsy (Lund, *et al.*, 1999; Harris, *et al.*, 2004a), which this study shows to be uncharacteristically low in DHA and other PUFA.

When using cadaveric tissue, Gudbjarnason *et al*., (1975, 1976, 1978), Belfrage *et al*., (1979), and Shenolikar (1980) under-reported many of the membrane phospholipid FA compared to those found in freshly obtained cardiac muscle. Sexton *et al*. (1995) was able to report a complete profile of phospholipid FA from cadaveric samples, however as with all cadaveric samples, analyses can be affected by autolysis during the waiting time for procurement.

Trends in cardiac membrane phospholipid FA across animal studies are difficult to interpret. Taken in light of animal species differences (Simon, *et al.*, 1969), the data required for comparisons are either not documented, or are missing on analysis, due to the differences in scientific questions and experimental methodologies; for example, early gas chromatographic (GC) techniques often did not allow sufficient run times to identify the very long-chain polyunsaturated fatty acids (such as DHA) or separation of DHA from very long chain saturates. As well, the specificity of GC column technology has greatly improved since those early reports and continues to do so. Whereas animal heart tissue is readily obtainable, procurement of human heart tissue is problematic, however due to human heart transplantation; this study provided a complete analysis of the membrane phospholipid FA composition of each chamber of normal, donor and failing, explanted human hearts. However a modest comparison of studies of un-supplemented animals found the atria and ventricles of marmoset monkeys and rat

compared favourably to DHA concentrations found in this human study. No further parallels can be found between animal data and the human data, apart from a reasonable conformity between animal and human hearts for the predominant n-3 PUFA of DHA, as well as the abundance of the n-6 PUFA, LA and AA, the SFA, PA and SA, and the MUFA OA in cardiac membranes. As with animals, ALA is found in near undetectable amounts, EPA is less than 1% of total and DPA remains relatively consistent.

The recent American Heart Association Science Advisory (AHA*, et al.*, 2009) states a diet of 5 to 10% of energy from n-6 PUFA, with an emphasis towards LA (~12 g for women and ~17 g for men) (Harris, 2010) as reducing the risk for CHD; recommended tissue and blood FA concentrations of LA and AA have not been given. The AHA advisory has been used by basic scientists in papers in attempting to understand the influence of diet on heart disease (Rennison*, et al.*, 2009) without full disclosure of a range of fatty acid concentrations; unlike Harris and von Schacky (Harris*, et al.*, 2004b) in determining acceptable ranges of red blood cell EPA+DHA concentrations for cardioprotection, >8% of total (for the omega-3 FAs, a healthy target intake is about 500 mg per day (whether from oily fish or fish oil capsules)). Understanding that there is competition between dietary n-6 PUFA and n-3 PUFA for the same desaturase enzymes, delta-6 (Δ^6) and delta-5 (Δ^5), for FA chain desaturation (the rate limiting step in carbon chain desaturation), increasing dietary n-6 PUFA will jeopardise the ability of the available dietary n-3 PUFA to synthesise longer chain PUFA from ALA.

The donor human hearts analysed in this study were regarded as normal, “healthy”, or viable (free of disease) and were initially intended for human heart transplantation. Further, these hearts from the broader community (free of overt heart disease) afforded a platform for analysis of cardiac membrane phospholipid FA compositions over the

course of the human lifespan. On the other hand, the explanted failing hearts were taken from a highly selected cardiac patient population, whose hearts have substantial underlying cardiac pathology and so provide a unique comparison to normal human cardiac membrane phospholipid FA profiles. These patients had no known clinical dietary intervention, therefore maintained a normal habitual diet too.

Focusing on the n-3 PUFA, DHA and EPA and regardless of group, this study has importantly demonstrated that over the course of the human lifespan, the fatty acids DHA and EPA+DHA increase in the membranes of cardiac muscle, in all four chambers; this is in line with an earlier observation of Gudbjarnason and Hallgrimsson (1975, 1978) of membrane phospholipid PUFA being "a function of age (and sex)". It was also demonstrated that with increasing age, the AA:DHA ratio decreased, corresponding further with Gudbjarnason and Hallgrimsson (1976). Hearts with very high AA:DHA ratios are proposed to be at a greater a risk of sudden cardiac death (Gudbjarnason*, et al.*, 1976); two explanted hearts were identified with obviously high AA:DHA ratios in the left atria, but these identified explanted hearts did not exhibit high ratios in other chambers. An explanation for this trend can be explained from the observations reported for the first time in this study. Such that, total PUFA was found to be significantly less in both atria and ventricles of explanted hearts when compared to donor hearts. This reflected significant reductions in LA, AA and total n-6 PUFA, and a modest reduction in the n-3 PUFA, DPA. In contrast the n-3 PUFA, DHA was found to be higher in explanted atria and ventricles, respectively. The data presented here show the differences in membrane phospholipid FA between normal and failing human hearts. The outstanding difference was the significantly greater concentration of DHA in failing ventricle (non-significant trend in atria) whereas all other PUFA were found in lower concentrations. The increase in DHA was further exemplified as a higher ratio of

DHA: DPA in all chambers, suggesting greater rate of conversion of long-chain n-3 PUFA. The increased myocardial DHA in failing hearts may represent an adaptive response of the cardiac muscle to the pathophysiological stress states. Animal studies indicate that DHA is increased after exposure to pressure overload (Reibel*, et al.*, 1986) and during catecholamine stress (Emilsson*, et al.*, 1983; Gudbjarnason, 1989; Gudbjarnason*, et al.*, 1995, 1996). Comparative physiology shows myocardial DHA concentrations increase in association with the well-established relationship between diminishing animal size and increasing metabolic rate and heart rate (Gudbjarnason*, et al.*, 1978a; Gudbjarnason*, et al.*, 1978c). In contrast, dietary induced increase in myocardial membrane DHA concentrations is associated with lower heart rate (McLennan*, et al.*, 2005), reduced catecholamine-induced arrhythmias (Gudbjarnason, 1989) and reduced hypertrophic response to pressure overload in controlled animal studies and in man is also associated with reduced heart rate (Geelen*, et al.*, 2005; Mozaffarian*, et al.*, 2005b), reduced sudden death (Marchioli*, et al.*, 2002) and reduced heart failure (Tavazzi*, et al.*, 2008a). Indeed a number of stress-induced changes in membrane DHA composition can be seen as adaptive responses since similar changes brought about by diet are cardioprotective (McLennan*, et al.*, 2005).

In the left side of the heart, which generates significantly greater atrial and ventricular pressures than the right side of the heart, the principle n-3 PUFA, DHA was found to have a more limited range in concentration than the right side of the heart in both donor and explanted hearts. Whether there is significance in this observation remains to be elucidated, but it is interesting to note that DHA concentrations in the right atria were the highest of all chambers; the chamber more likely to be biopsied and used for comparisons to red blood cell membrane phospholipid FA EPA+DHA (RBC EPA+DHA). Also known as the Omega-3 Index (Harris*, et al.*, 2004b), RBC

EPA+DHA is an index which can be used as a biomarker and possibly a risk factor for heart health in humans; RBC EPA+DHA ≥8% - superior cardioprotection, ≤4% - reduced cardioprotection.

Sex differences are seen in heart membrane phospholipid FA composition and heart function and dysfunction (Du*, et al.*, 2006), which can be attributed to the diet (Slater-Jefferies*, et al.*, 2010), sex hormones (Du*, et al.*, 2006; Childs*, et al.*, 2008), liver uptake (Luxon*, et al.*, 1998) and the recently identified differences in human cardiac stem cells (hCSCs) (Kajstura*, et al.*, 2010). The study participants here were principally male in both explant and donor hearts. The small number of female hearts in this study would preclude definitive conclusions to be drawn between the sexes. However, close approximations of FA concentrations in males and females was observed by Rocquelin *et al*., (1985), moreover males are more likely to be affected by heart disease than pre-menopausal women due to the lack of any oestrogenic protection (Du*, et al.*, 2006). Substantially most papers conferring human heart membrane fatty acid data have limited female participation; Garg *et al*., (2006) 6% were female, Metcalfe *et al*., (2007, 2010) had 10% and 20% female participants respectively, and Harris *et al*., (2004) had 25% female study participation. The mechanistic significance of DHA increasing in cardiac tissues with age is yet to be fully elucidated, yet in aging hearts increased DHA may well be an adaptive response (Pepe*, et al.*, 2002) as in other cardiac stress.

2.4. CONCLUSION

This study has provided an opportunity to establish the fatty acid composition of human heart in freshly obtained tissue using collection, storage and analysis methods that are the same as commonly used for animal dietary intervention studies. Previous studies

with membrane phospholipid fatty acid analysis of the human heart have examined tissue that was obtained from cadavers of variable and indeterminate time of death (Fletcher, 1972; Gudbjarnason*, et al.*, 1975a; Sexton*, et al.*, 1995) or very small intra-ventricular biopsy (Harris*, et al.*, 2004a). The study revealed that amongst the n-3 PUFA, EPA was present at very low concentrations, consistently less than 1% of the fatty acids in any heart chamber. Docosahexaenoic acid was shown to be consistently greater than ALA, EPA or DPA in all chambers, with AA and LA the predominant n-6 PUFA. Docosahexaenoic acid and EPA but no other PUFA, changed with age, as shown by the reduced AA:DHA ratio. There was no difference in age between donor and explant hearts. Comparison of chambers showed total n-3 PUFA to be greater in the atrium than the ventricles and total n-6 PUFA to be greater in the ventricles than atrium. The results indicate that DHA is the principle n-3 PUFA in the human (as seen in animal) heart and that it increases with age and under stress as seen in laboratory animals. This data provides a baseline for evaluation of human heart samples taken by biopsy within dietary studies.

Chapter 3

FATTY ACID COMPOSITION OF RED BLOOD CELL MEMBRANES AS A MARKER OF HUMAN HEART MEMBRANE PHOSPHOLIPID FATTY ACIDS

3.0. INTRODUCTION

The n-3 PUFA are essential to the human diet, due to the inability of mammals to synthesize these fatty acids *de novo*. Desaturase and elongase enzymes are capable of altering the precursor fatty acids, EPA and alpha-linolenic acid (ALA, 18:3n-3) into the long-chain DHA, albeit in a limited fashion (Arterburn*, et al.*, 2006) as the disparity between amount consumed (ALA approximately 1.2 *g*/day in Australia) and tissue concentrations attest (typically < 0.5% of total fatty acids) (Burdge*, et al.*, 2005). There is a need therefore for preformed EPA and DHA to be consumed (Arterburn*, et al.*, 2006; Wang*, et al.*, 2006; Milte*, et al.*, 2008) and dietary guidelines from numerous countries with low EPA and DHA consumption (NHMRC, 2003; WHO/FAO, 2004; AHA*, et al.*, 2006; Harris*, et al.*, 2008a; NHFA, 2008b) recommend at least two oily fish meals (approximately 220 *g* total) per week, in addition to an overall healthy diet and lifestyle plan, to help reduce heart disease risk (WHO, 2003; AHA*, et al.*, 2006; AHA*, et al.*, 2009).

Extensive human and animal studies examining the ingestion of the long-chain n-3 PUFA, eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acids (DHA, 22:6n-3), have identified multiple mechanisms by which these fatty acids may reduce risk of death from heart and vascular diseases. Foremost in this research is the antiarrhythmic effect of omega-3 PUFA, principally DHA, whose incorporation into the membranes of heart phospholipid fatty acids directly affect cellular electrophysiology (for review see (McLennan, 2004)). Interest is growing in the role of DHA as the forerunner for cardiovascular (CV) health due to its abundance and its avid incorporation into heart cell membranes, graphically shown in rats in a time- and dose-dependent manner (Owen*, et al.*, 2004; Slee*, et al.*, 2010). However, as species differences for membrane

phospholipid fatty acids clearly exist (Chapter 1, Table 1.4) (Jordan, *et al.*, 2004), it remains to be seen whether similar relationships between dietary fatty acids and membrane fatty acid composition holds for the human heart.

Animal studies have provided numerous examples of dietary n-3 PUFA incorporation into the membranes of myocardial cells (Gudbjarnason, 1975, 1980; Charnock, *et al.*, 1983; Charnock, *et al.*, 1985a; Owen, *et al.*, 2004; Slee, *et al.*, 2010) and into other cell types. Blood plasma, platelets, leukocytes and erythrocytes (red blood cells, RBC) have all been used to identify dietary trends of fatty acid intakes. Plasma serves as an indicator of acute dietary intake, whereas RBC membrane phospholipids may act as a marker of the more consistent recent diet (Brown, *et al.*, 1991; Cao, *et al.*, 2006). Notably, DHA has been identified as having a time- and dose-dependent effect on RBC membrane FA composition in humans (Milte, *et al.*, 2008). However, different tissues including the heart, cerebral cortex, retina, liver and kidneys in animals (Stubbs, *et al.*, 1984; Saito, *et al.*, 1998; Owen, *et al.*, 2004) and in humans: sperm, adipose tissue, buccal epithelial cells, spleen, rectal epithelium and skeletal muscle (Arterburn, *et al.*, 2006) often display large differences in membrane phospholipid fatty acid composition, particularly in relation to DHA concentrations. Nevertheless, RBC membranes in blood samples from people who died of sudden cardiac death (SCD) having no prior known heart disease, were more likely to be low in DHA and EPA than healthy age-matched controls, and in turn, a low dietary fish intake was associated with these primary cardiac arrest victims (Siscovick, *et al.*, 1995).

Noting a pattern in the association between RBC fatty acid composition and the primary cardiac arrest and other heart disease outcomes, Harris and von Schacky proposed the EPA+DHA content of RBC membranes as a new risk factor for cardiac disease, which they termed the Omega-3 Index (Harris, *et al.*, 2004b). However, it is unlikely that the

RBC fatty acid composition itself or any associated RBC functional change is directly responsible for the changes in cardiovascular risk; therefore this "Index" is thought to act as a marker of heart fatty acid composition. The collection of blood samples is a relatively non-invasive means of assessing recent consumption of dietary fats, whereas heart sampling is invasive and dangerous and usually only possible if coincidental with intrathoracic surgery, most commonly from the atrium of ailing hearts. Yet, as described above, considerable tissue differences exist in n-3 PUFA composition (Arterburn*, et al.*, 2006), and with limited reports identifying the incorporation of dietary EPA and DHA into the membranes of human cardiomyocytes (Harris*, et al.*, 2004a; Garg*, et al.*, 2006; Metcalf*, et al.*, 2007) (Table 3.1) it is not established how well RBC act as a marker of human heart tissue.

Thus, the aim of this study was two-fold: first to characterise and contrast the membrane phospholipid fatty acid composition of the human atrium and RBC paired from the same subjects and obviously exposed to the same dietary patterns; and secondly, to examine the influence of fish oil (FO) supplementation on atrial and RBC membranes composition, also paired from the same subjects, and contrast them to subjects supplemented with placebo.

Table 3.1. Comparison of the Major Membrane Phospholipid Fatty Acids[1] from Human Atria and Red Blood Cells after Fish Oil Supplementation or Control Diets.

CARDIAC Membrane Fatty Acids			n-6 PUFA		n-3 PUFA						
Reference	Diet	N	LA	AA	EPA	DPA	DHA	EPA+DHA	Total PUFA	Total SFA	Total MUFA
*Harris, 2004**	*C*	*21*	*[2]9*	*9*	*0.2*	*0.8*	*1.5*	*1.7*			
Garg, 2006	C	9	17	22	0.8	2	6.7	7.4		35	15
Metcalf, 2007	C	10	14.7	20.5	0.5	2	4.8	5.3	30	40	12.6
*Harris, 2004**	F.O.	*21*	*8*	*8*	*0.6*	*0.8*	*2.3*	*3*			
Garg, 2006	F.O.	8	17	18	3	2	8	11.4		35	14
Metcalf, 2007	F.O.	10	13.8	17.5	1.4-3	2	6.8-8.5	8.2-11.5	30.8	38.8	13.2

RED BLOOD CELL Membrane Fatty Acids			n-6 PUFA		n-3 PUFA						
Reference	Diet	N	LA	AA	EPA	DPA	DHA	EPA+DHA	Total PUFA	Total SFA	Total MUFA
Harris, 2004	C		10	17	0.4	2.7	4.2	4.7			
Metcalf, 2007	C	10	8.5	14.2	0.7	3	4.4	5.1	28.9	42.5	18.6
Harris, 2004	F.O.	21	9	15	1.5	3.9	7.5	9			
Metcalf, 2007	F.O.	10	6.9	14.1-11.7	1.9-3.1	3	5.7-7.6	7.7-10.7	30.4	42.8	18.5

* Harris *et al.*, 2004 cardiac samples were right intraventricular biopsy from ≥ 3 months post-heart transplant.[1] Results are a percentage of total fatty acids. [2] Values have been averaged. C - Control, F.O. - Fish Oil, AA - arachidonic acid; LA - linoleic acid; EPA - eicosapentaenoic acid; DPA- docosapentaenoic acid; DHA - docosahexaenoic acid; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.

3.1. METHODS

3.1.1. SAMPLES

The subjects were patients scheduled for elective on-pump cardiac surgery (valve repair or replacement or coronary artery bypass grafting (CABG)) at the Alfred Hospital, Melbourne between March 2005 and August 2006. Thirty four subjects were randomised into two groups at the time of preadmission clinic; the Placebo group consumed olive oil (3 x 1 g) capsules and the fish oil (FO) treatment group, (3 x 1 g) capsules per day. The FO supplementation delivered 540 mg EPA + 360 mg DHA per day. Supplementation commenced on the day of the pre-admission clinic and continued until the day of surgery, with treatment duration ranging from 13 to 93 days (mean = 42.8 days). Baseline blood samples were provided for 23 subjects, taken at their pre-admission clinic. From a total number of n=31 subjects, we obtained peri-operative blood samples and right atrial samples were obtained from 28 subjects. Paired baseline and peri-operative blood samples were obtained from n=11 placebo and n=10 FO subjects and paired peri-operative blood samples and right atrial samples were obtained from n=14 placebo and n=13 FO subjects. Red blood cells and atrial samples were obtained, handled and treated as per institute protocols. Sample handling faults and transport failure accounted for the discrepancies in total and matched samples. Informed consent was obtained before participation in the study, which was approved by the Research Ethics Committee of the Alfred Hospital and Monash University Clinical Trial registration number NCT00906646 (www.clinicaltrials.gov).

3.1.2. SAMPLE PREPARATION AND FATTY ACID ANALYSIS

3.1.2.1. Human Red Blood Cell Preparation

i) RBC Membranes (Ghosts)

Red blood cells were stored at -80 °C until analysis, when the RBC samples were thawed on ice and maintained on ice when possible throughout procedures. At the commencement of RBC analysis, 400 µL of packed RBC where transferred to clean and dry 10.4 mL screw-cap transparent polycarbonate ultracentrifuge bottles (Beckman-Coulter Australia Pty Ltd, Gladesville, NSW, part number: 355603). Addition of 10 mM Tris-HCl buffer, pH 7.4, was added to RBC sample to a weight of between 16.3 – 16.5 g including bottle caps and O-rings. The capped tubes were gently inverted and left at room temperature for 30 – 40 minutes to completely lyse cells.

The samples were then spun at 3000 *g* ultracentrifugation (Beckman-Coulter Optima L-100, fixed-angle, titanium rotor, type 70.1 Ti, part number: 342184) in vacuum at 4 °C for 30 minutes. The haemoglobin tainted aqueous solution was aspirated to leave RBC membrane pellet which was washed gently with 0.5 mL of 10 mM Tris-HCl buffer, this was also aspirated. After the addition of 200 µL distilled water, the pellet was resuspended using a positive displacement pipette, avoiding foam to form, and allowed to stand for several minutes.

ii) RBC Direct Transesterification

Red blood cell membrane phospholipid fatty acids were methylated to phospholipid fatty acid methyl esters (FAME) in preparation for gas chromatography. From the resuspended RBC membrane ghosts for each subject, 150 µL was transferred to individual clean 7 mL glass screw-cap test-tubes, after which 2 mL methanol: toluene (4:1, v:v) was added.

In a fume hood whilst vortexing, 200 μL of acetyl chloride was carefully added. Taking care of the increase in temperature, the tubes were securely capped with temperature resistant caps and Teflon ^{TM} tape wrapped around thread of tube. The tubes were placed in a dry heat block for 60 minutes at 100 °C, after which the tubes were immediately placed in ice for rapid cooling to cease further reactions. The cold tubes and samples were vortexed whilst 5 mL of refrigerator cold 6% K_2CO_3 from a bottle-top dispenser was added. Recapping tubes and after shaking vigorously for 30 seconds, the tubes were placed in a refrigerated benchtop centrifuge at 2000 *g* for 10 minutes at 0 °C. The separation of lipid FAMEs from the aqueous phase was achieved.

The upper lipid phase was transferred carefully into 250 μL glass inserts (catalogue number: 24701; glass inserts, shell style 0.25 mL 5mm x 31 mm, outside diameter x height: Sigma-Aldrich/Supelco, Bellefonte, PA) which were placed in 2 mL glass standard opening vials (catalogue number: 27078; 2.0 mL outside diameter clear vial, 8-425 thread, 12 mm in diameter, 32 mm in height, and has a 4.6 mm opening: Sigma-Aldrich/Supelco), and tightly capped with septa (catalogue number: 27079-U; silicone septa, red Teflon ^{TM}-faced, 8 mm: Sigma-Aldrich/Supelco) and caps (catalogue number: 5076670; holed white polypropylene caps, 8-425 thread size: Sigma-Aldrich/Supelco).

3.1.2.2. Human Atrial Sample Preparation

Right atrial samples were stored at until ready for shipping in dry ice, stored again at -80 °C prior to analysis, and kept ice-cold when possible during initial processes of analysis. Briefly, tissue samples (atrium: 20 - 50 mg wet weight) were prepared by removing obvious fatty deposits prior to homogenisation. A modified Folch (Folch*, et*

al., 1956) lipid extraction technique was employed. As detailed previously (Chapter 2, 2.2. METHODS), the samples were treated equally throughout all processes of Phospholipid Fraction Isolation, Fatty Acid Methyl Ester (FAME) Formation, FID-Gas Chromatography and Quantification of Fatty Acids Methyl Esters.

iii) Fatty Acid Methyl Ester Formation

Briefly, all atrial membrane phospholipid fatty acids were methylated to phospholipid fatty acid methyl esters (FAME) in preparation for gas chromatography. The derived lipid samples were collected into clean screw-top tubes, placed in a heat block (average temperature of 25 °C) and dried down under nitrogen gas to pure lipid, to which 2 mL methanol: toluene (4:1, v/v) was added. In a fume hood, whilst vortexing, 200 µL acetyl chloride was added, the samples where then placed in the heat block for 60 minutes at 100 °C. At the end of 60 minutes the samples were quickly removed from the heat block and rapidly cooled in ice, to then be centrifuged at 4000 *g* for 10 minutes. The resultant lipid layer was then pipette into clean deactivated borosilica glass vials, using 250 µL glass inserts.

iv) Gas Chromatography and Quantification of Fatty Acid Methyl Esters

The fatty acid methyl esters were analysed by FID-GC (flame ionizing detector - gas chromatography) using a Shimadzu GC-17A with AOC-20i (automated sample injection system) with a 50 m (length) x 0.25 µm (wall coating) x 0.25 mm (internal diameter) CP-Select^{TM} CB for FAME capillary column (Varian, Middleburg, The Netherlands. Catalogue number: CP7419). Each sample run consisted of 10 to 25 samples with two FAME reference standards utilised (NuChek Prep, Elysian, MN, USA, catalogue number: GLC 673B and Sigma-Aldrich Qualitative F.A.M.E. Mix, C4-C24, Catalogue number: 18919-1AMP). A 1 µL sample was injected into the split

system using hydrogen as the carrier gas, with a temperature ramp function commencing at 170 °C and rising to 210 °C over 24 minutes.

3.1.3. CHEMICALS

All chemicals, solvents and laboratory consumables are of analytical grade supplied by Crown Scientific Pty Ltd (Ebos Group Ltd., Minto, NSW), Sigma-Aldrich Corporation (Castle Hill, NSW), Phenomenex Australia Pty Ltd (Pennant Hills, NSW), BOC Gases Australia Ltd (North Ryde, NSW), Waters Corporation Australia (Rydalmere, NSW), Wheaton Science Products (Wheaton Industries Inc., Millville, NJ, USA) and NuChek Prep Inc. (Elysian, MN, USA).

3.1.4. STATISTICAL ANALYSIS

Data was analysed using Statistix (version 9, Analytical Software, Tallahassee, FL, USA) and graphed using GraphPad Prism (version 6, GraphPad Software Inc., USA). Data are presented as mean ± SEM. The RBC baseline v RBC peri-operative and RBC peri-operative v right atrium was analysed within treatment groups by paired, repeated measures ANOVA. Between treatments effects were analysed by one way ANOVA within each sample type (baseline RBS; peri-operative RBC and atria). Linear regression and correlation analyses were conducted on individual fatty acids between sample types. Statistically different results were accepted with $p<0.05$.

3.2. RESULTS

3.2.1. HUMAN RED BLOOD CELLS

3.2.1.1. Control

Baseline red blood cell membrane phospholipids included from all subjects were characterised by total SFA of 42.3±1.5% (mean ± standard deviation), total MUFA of 18.8±2.0% and total PUFA of 34.6±2.0%. The important n-6 PUFA, LA and AA were present at 7.1±0.9% and 14.5±1.3% of total fatty acids respectively, with 2.8±0.6% 22:4n-6 and total n-6 PUFA of 26.2±2.0%. The major n-3 PUFA of interest, EPA and DHA, were present at 0.77±0.26% and 4.89±1.29% respectively, with 2.54±0.24% DPA and total n-3 PUFA of 8.22±1.24%. The baseline sum of EPA+DHA in red blood cells was 5.67±1.31%. All fatty acids of interest are listed in Tables 3.2 & 3.3. There were no differences in any individual fatty acid or subgroup between the placebo or FO supplemented subjects at baseline.

3.2.1.2. Effects of Placebo or Fish Oil Supplementation

There were no differences in any individual fatty acid percent concentration or subgroup between the peri-operative blood sample and baseline within the placebo supplemented subjects (Table 3.2). Subjects with FO supplementation had significantly higher concentrations of EPA, DHA and DPA, with resultant increases in sum of EPA+DHA and total n-3 PUFA in their peri-operative blood samples. There were corresponding decreases in the n-6 PUFA LA, AA and 22:4n-6 with a resultant decrease in total n-6 PUFA. All of these differences were observed both between placebo and peri-operative FO samples and within the FO group between baseline and peri-operative samples (Table 3.3). There were no differences in individual or total SFA or MUFA or in total PUFA with FO supplementation.

Table 3.2. Comparison of major phospholipid fatty acids from human red blood cells and atria: Placebo.

PLACEBO	Baseline RBC	Peri-operative RBC	Atria	Baseline RBC versus Peri-operative RBC *P*	Peri-operative RBC versus Atria *P*
Fatty acid[1] N =	13	17	14	(n=11)	(n=14)
16:0 palmitic	19.80 ± 0.40	19.86 ± 0.23	23.08 ± 2.03	*n.s.*	*n.s.*
18:0 stearic	14.96 ± 0.23	14.99 ± 0.21	15.83 ± 0.68	*n.s.*	*n.s.*
Total SFA	42.26 ± 0.44	42.80 ± 0.52	40.12 ± 1.59	*n.s.*	*n.s.*
18:1 n-9 oleic	11.46 ± 0.36	11.15 ± 0.24	10.61 ± 0.53	*n.s.*	*n.s.*
Total MUFA	18.24 ± 0.73	18.23 ± 0.22	15.81 ± 0.67	*n.s.*	* *<0.001*
18:2 n-6 (LA)	7.14 ± 0.23	7.38 ± 0.27	16.58 ± 1.05	*n.s.*	* *<0.0001*
20:4 n-6 (AA)	14.76 ± 0.33	14.81 ± 0.30	18.55 ± 0.90	*n.s.*	* *<0.0001*
22:4 n-6	2.91 ± 0.18	2.93 ± 0.13	0.52 ± 0.07	*n.s.*	* *<0.0001*
22:5 n-6	0.38 ± 0.05	0.38 ± 0.03	0.40 ± 0.21	*n.s.*	*n.s*
Total n-6 PUFA	26.64 ± 0.44	26.92 ± 0.37	36.51 ± 1.77	*n.s.*	* *<0.0001*
18:3 n-3 (ALA)	0.04 ± 0.02	0.04 ± 0.02	0.11 ± 0.01	*n.s.*	* *<0.001*
20:5 n-3 (EPA)	0.70 ± 0.06	0.74 ± 0.05	0.28 ± 0.08	*n.s.*	* *<0.0001*
22:5 n-3 (DPA)	2.56 ± 0.07	2.56 ± 0.07	1.47 ± 0.11	*n.s.*	* *<0.0001*
22:6 n-3 (DHA)	4.89 ± 0.28	4.84 ± 0.22	4.54 ± 0.29	*n.s.*	*n.s.*
EPA+DHA	5.59 ± 0.25	5.58 ± 0.20	4.82 ± 0.28	*n.s.*	* *0.05*
Total n-3 PUFA	8.17 ± 0.24	8.15 ± 0.19	6.39 ± 0.33	*n.s.*	* *0.002*
Total PUFA	35.00 ± 0.57	35.23 ± 0.40	43.04 ± 2.01	*n.s.*	* *<0.0001*

Phospholipid fatty acid analysis of red blood cell (RBC) samples at baseline and peri-operatively and from atria. Peri-operative samples taken after placebo oil supplementation. [1] % of total fatty acids (means±SEM). AA, arachidonic acid; ALA, α-linoleic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.* Significantly different Baseline RBC v Peri-operative RBC paired samples: repeated measures ANOVA (n= 11) and Peri-operative RBC to Atria paired samples: repeated measures ANOVA (n=14). n.s.: not significant (*P>0.05*). n.d.: not detected

Table 3.3. Comparison of major phospholipid fatty acids from human red blood cells and atria: FO supplemented.

FO	Baseline RBC[2]	Peri-operative RBC	Atria	Baseline RBC versus Peri-operative RBC *P*	Peri-operative RBC versus Atria *P*
Fatty acid [1] n =	10	14	14	(n=10)	(n=13)
16:0 palmitic	20.27 ± 0.36	20.10 ± 0.33	22.46 ± 1.50	*n.s.*	*n.s.*
18:0 stearic	14.71 ± 0.26	14.68 ± 0.15	14.87 ± 0.41	*n.s.*	*n.s.*
Total SFA	42.35 ± 0.47	42.67 ± 0.32	38.13 ± 1.43	*n.s.*	* *0.015*
18:1 n-9 oleic	12.25 ± 0.47	11.95 ± 0.37	11.41 ± 0.26	*n.s.*	*n.s.*
Total MUFA	19.51 ± 0.49	19.14 ± 0.42	16.31 ± 0.60	*n.s.*	* *<0.001*
18:2 n-6 (LA)	7.09 ± 0.33	†† 6.35 ± 0.19	16.00 ± 0.76	* *0.05*	* *<0.0001*
20:4 n-6 (AA)	14.21 ± 0.44	†† 13.18 ± 0.34	19.28 ± 0.78	* *0.006*	* *<0.0001*
22:4 n-6	2.70 ± 0.16	†† 2.37 ± 0.12	0.36 ± 0.05	* *0.018*	* *<0.0001*
22:5 n-6	0.29 ± 0.07	0.44 ± 0.22	0.17 ± 0.13	*n.s.*	*n.s.*
Total n-6 PUFA	25.74 ± 0.75	†† 23.67 ± 0.54	36.24 ± 1.14	* *0.003*	* *<0.0001*
18:3 n-3 (ALA)	n.d ±	0.08 ± 0.05	0.05 ± 0.05	*n.s.*	*n.s.*
20:5 n-3 (EPA)	0.87 ± 0.10	†† 1.57 ± 0.12	†† 0.95 ± 0.19	* *0.0002*	* *0.018*
22:5 n-3 (DPA)	2.51 ± 0.07	† 2.90 ± 0.12	1.49 ± 0.15	* *0.016*	* *<0.0001*
22:6 n-3 (DHA)	4.91 ± 0.54	† 5.94 ± 0.48	† 6.03 ± 0.50	* *0.008*	*n.s.*
EPA+DHA	5.77 ± 0.56	†† 7.50 ± 0.51	† 6.99 ± 0.67	* *0.0004*	*n.s.*
Total n-3 PUFA	8.29 ± 0.53	† 10.42 ± 0.45	† 8.52 ± 0.77	* *0.0002*	* *0.020*
Total PUFA	34.14 ± 0.59	34.17 ± 0.41	45.09 ± 1.71	*n.s.*	* *<0.0001*

Phospholipid fatty acid analysis of red blood cell (RBC) samples at baseline and peri-operatively and from atria. Peri-operative samples taken after supplementation with FO. AA, arachidonic acid; ALA, α-linoleic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. [1] % of total fatty acids (means±SEM). [2] Note no significant differences in RBC: baseline FO compared to Placebo. † Significantly different to corresponding Placebo samples (Table 3.2.) - ANOVA: † *P < 0.05*, †† *P<0.005*. * Significantly different Baseline RBC to Peri-operative RBC paired samples: repeated measures ANOVA (n= 10) and Peri-operative RBC sample to Atria paired samples: repeated measures ANOVA (n=13). n.s.: not significant (*P>0.05*). n.d.: not detected

3.2.2. HUMAN RIGHT ATRIA

3.2.2.1. Control

Human atrial samples from placebo supplemented subjects were characterised by total SFA of 40.1±5.9% (mean ± standard deviation), total MUFA of 15.7±2.4% and total PUFA of 43.0±7.5%. The important n-6 PUFA, LA and AA were present at 16.6±3.9% and 18.6±3.4% of total fatty acids respectively, with total n-6 PUFA of 36.5±6.6%. The major n-3 PUFA of interest, EPA and DHA, were present at 0.28±0.32% and 4.54±1.10% respectively, with total n-3 PUFA of 6.39±1.25%. The sum of EPA+DHA in the atria was 4.82±1.06%. All fatty acids of interest are listed in Table 3.2.

3.2.2.2. Effects of Fish Oil Supplementation

Subjects with FO supplementation had significantly higher concentrations of EPA and DHA with resultant increases in sum of EPA+DHA and total n-3 PUFA in their atrial samples compared to placebo supplemented subjects (Table 3.3). There were no other significant differences in individual or total SFA, MUFA or n-6 PUFA or total PUFA between the FO and placebo supplemented atria.

3.2.3. HUMAN RED BLOOD CELL COMPARED TO HUMAN ATRIUM

Atria were characterized by higher concentrations of the n-6 PUFA LA and AA and total n-6 PUFA (especially LA, which was more than doubled) compared to their paired peri-operative blood samples (Tables 3.2 & 3.3). There were corresponding significantly lower concentrations of the n-3 PUFA EPA, DPA, EPA+DHA and total n-3 PUFA in the atria compared with red blood cells. Total PUFA was higher in atria, the total MUFA was lower and there was a trend to lower SFA which was significant in FO subjects. In the atria there was a significantly higher ratio of n-3 PUFA DHA/DPA

(atria 3.39 ± 0.41; RBC 1.93 ± 0.12) and a lower ratio of n-6 PUFA 22:4/AA (atria 0.027 ± 0.003; RBC 0.198 ± 0.008) and AA/LA (atria 1.14 ± 0.05; RBC 2.06 ± 0.10) (Figure 3.1. Placebo).

With FO supplementation there were additional reductions in total SFA in the atria and DHA concentration was significantly higher than in the red blood cells with no overall significant difference in EPA+DHA between atria and red blood cells, but otherwise the major differences between atria and RBC were seen commonly, irrespective of supplementation (Tables 3.2 & 3.3). The greater increase in DHA in FO atria relative to RBC resulted in further increase in the DHA/DPA ratio, whereas the other ratios were unchanged (Figure 3.1, FO).

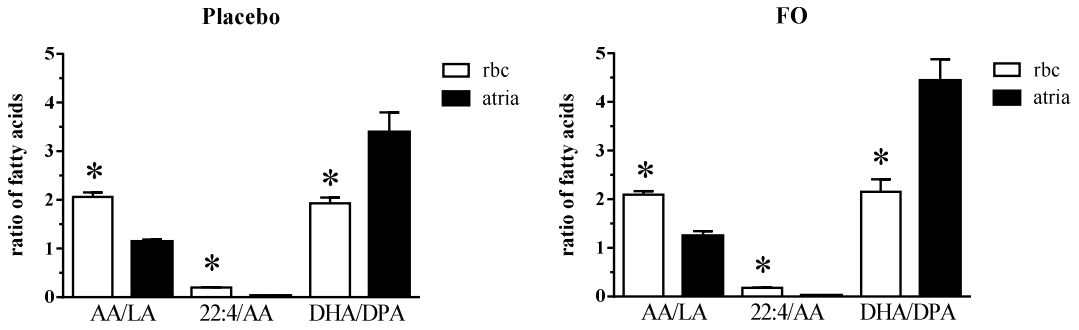

Figure 3.1. Ratios of specific n-6 and n-3 PUFA within membranes of red blood cells (RBC) and atria of Placebo and FO supplemented subjects.
The increases in n-3 PUFA and decreases in n-6 PUFA were reflected in significantly lower ratios of (LA+AA)/(EPA+DHA) in FO atria (5.56±0.45) and RBC (2.77±0.21). * RBC significantly different to atria

3.2.4. CHANGES TO N-3 PUFA IN HUMAN RBC AND ATRIA MEMBRANE PHOSPHOLIPID FATTY ACIDS FROM FISH OIL TREATMENT

The differences in n-3 PUFA composition between placebo and FO supplemented subjects were most evident as increases in EPA and DHA (Figure 3.2). In red blood cells, there were similar increases in both EPA and DHA, whereas in atria the difference in DHA was almost twice that of EPA. There were no changes in tissue DPA or ALA with FO supplementation.

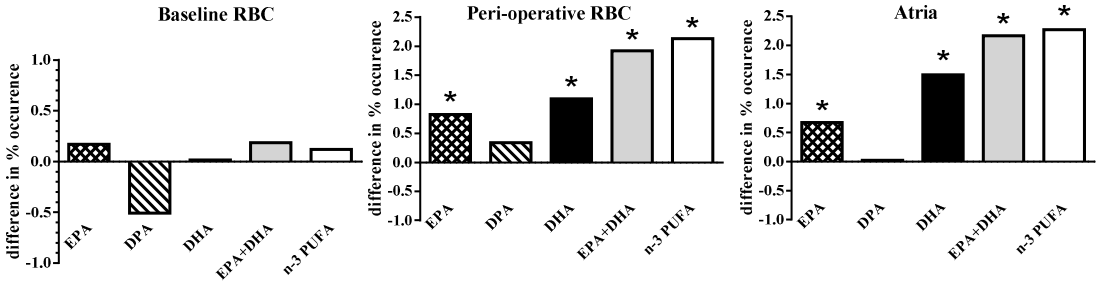

Figure 3.2. Baseline and effect of FO supplementation on individual and summed n-3 PUFA in RBC and atria: Difference between Placebo and FO.
* FO significantly different to Placebo (0% difference: FO = Placebo)

3.2.5. CORRELATIONS BETWEEN FATTY ACIDS IN RBC AND ATRIA

Paired samples from all subjects (N=26) demonstrated a significant correlation between RBC membrane EPA+DHA and atrial membrane EPA+DHA (Figure 3.4., D) (r^2=0.4286). Indications of weaker associations were shown for other n-3 PUFA and combinations in paired RBC and atrial samples (Table 3.4).

Table 3.4. Associations between RBC n-3 PUFA and Atria n-3 PUFA[1].

RBC	Atria	r^2	*P* for slope	Placebo N=13	FO N=13
EPA	EPA	0.3009	0.005	n.s.	n.s.
EPA	DHA	0.3196	0.003	n.s.	n.s.
EPA	EPA+DHA	0.3228	0.003	n.s.	n.s.
EPA	EPA+DPA+DHA	0.2729	0.007	n.s.	n.s.
EPA+DHA	DHA	0.3112	0.003	n.s.	n.s.
EPA+DHA	DPA	0.0127	n.s.	0.018	n.s.
EPA+DHA	EPA+DHA	0.4286	0.0004	n.s.	n.s.
EPA+DPA+DHA	EPA+DPA+DHA	0.2243	0.017	n.s.	n.s.
DHA	DHA	0.1955	0.024	n.s.	n.s.(0.06)
DHA	DPA	0.0220	n.s.	0.019	n.s.
DHA	EPA+DHA	0.3136	0.004	n.s.	n.s.
DHA	EPA+DPA+DHA	0.1481	0.057	n.s.	n.s.
DPA	DPA	0.0078	n.s.	n.s.	n.s.
DPA	DHA	0.0063	n.s.	0.002	n.s.
RBC	**RBC**				
EPA	DPA	0.1146	n.s.(0.07)	n.s.	n.s.
DPA	DHA	0.0503	n.s.	n.s.(0.052)	n.s.(0.076)
Atria	**Atria**				
DPA	DHA	0.0220	n.s.	0.019	n.s.

[1] Except for RBC v RBC; Atria v Atria. Linear regression analysis performed on tissue fatty acids, Significant results ($p<0.05$) are shown. n.s. not significant.

In the figure following (Figure 3.3) neither RBC EPA (A, C) alone, RBC DHA (B) alone, nor RBC DPA (D) alone correlates as well as RBC EPA+DHA (Figure 3.4, D) does with atrial EPA+DHA. Placebo and FO RBC EPA, and Placebo and FO RBC DHA, have trends to positive atrial association with EPA and atrial DHA respectively. However, there are negative trends seen for association between (i) Placebo RBC EPA and (ii) Placebo RBC DPA (significantly negative p=0.002), and atrial DHA.

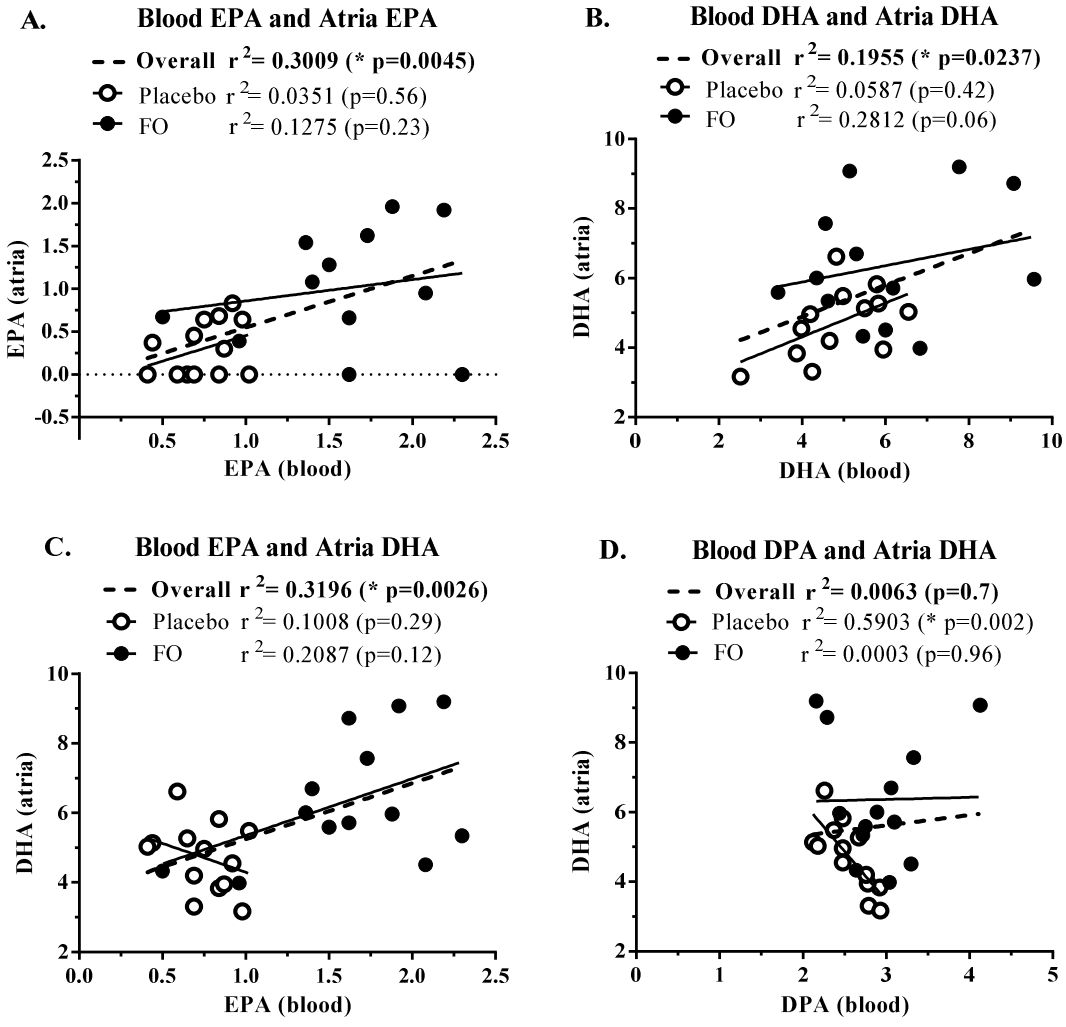

Figure 3.3. Graphs A - D show associations of RBC EPA, DHA and DPA to Atria EPA and Atria DHA.

* statistically significant; --- linear regression line for combined Placebo and FO groups

In the figure following (Figure 3.4) neither RBC EPA (A) alone, RBC DHA (B) alone, nor RBC EPA+DPA v RBC DHA (C) correlates as well as RBC EPA+DHA does with atrial EPA+DHA (Figure 3.4, D).

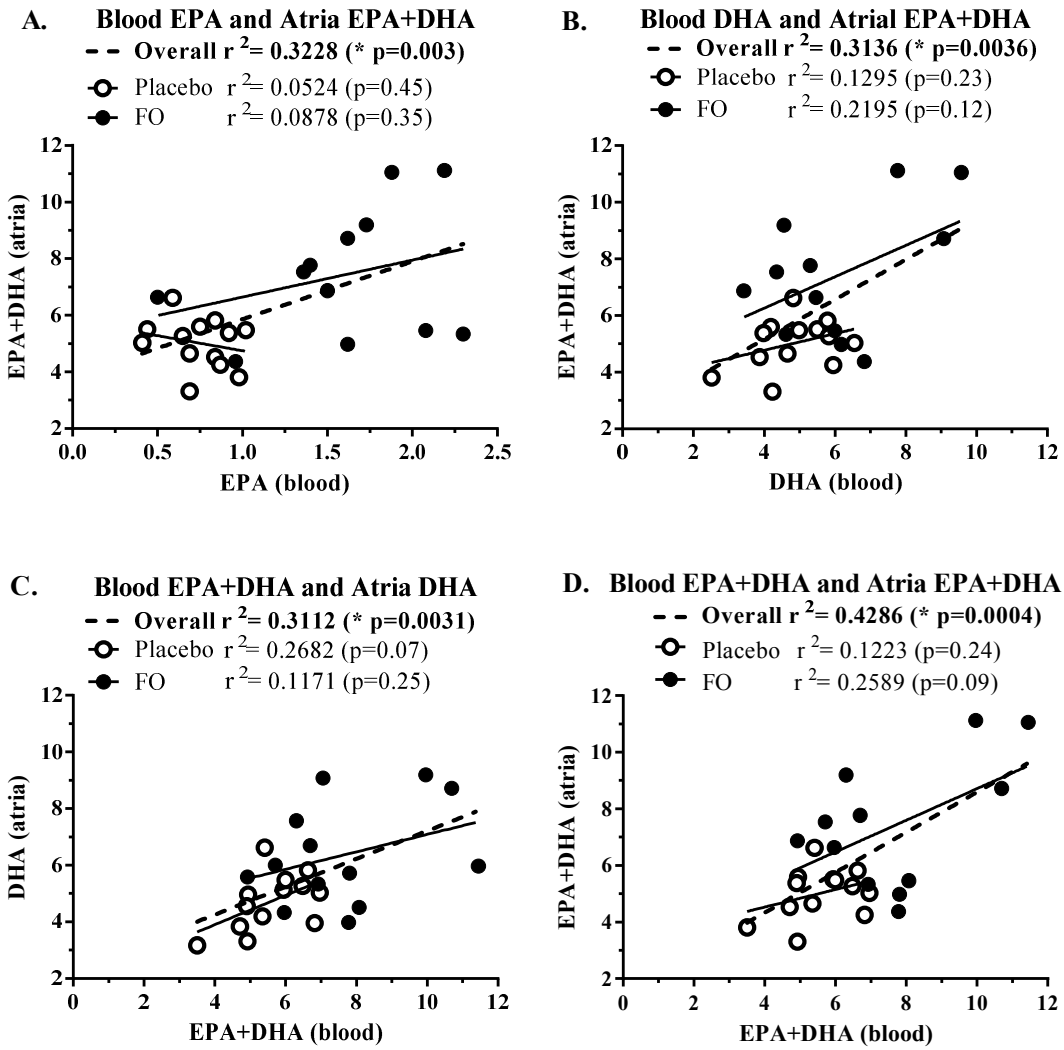

Figure 3.4. Graphs A, B, C and D associations are shown of RBC EPA, DHA and EPA+DHA to Atria EPA+DHA and Atria DHA.

* statistically significant; ---linear regression line for combined Placebo and FO groups

In figure following (Figure 3.5) RBC DPA has no significant correlation with RBC DHA (A), atrial DPA (B), nor Atrial EPA+DPA+DHA (C). The Placebo supplemented group trends to lower DHA in RBC and Atria with greater RBC DPA. DPA has no significant correlation with RBC EPA+DHA.

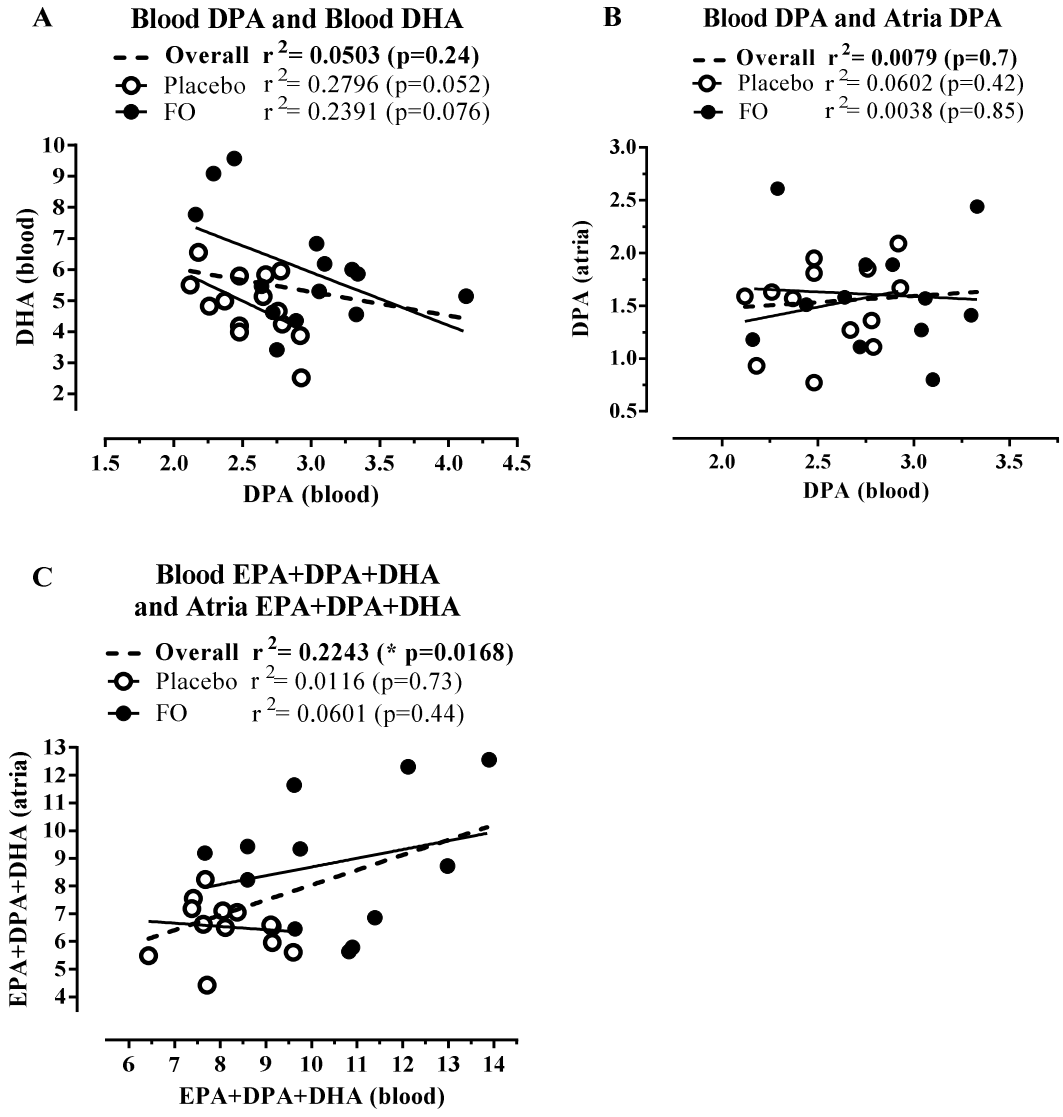

Figure 3.5. Associations of the long-chain n-3 PUFA in red blood cell and atria.

A. RBC DPA and RBC and DHA. **B.** RBC DPA and Atria DPA and **C.** RBC EPA+DPA+DHA and Atria EPA+DPA+DHA.

* statistically significant. ---linear regression line for combined Placebo and FO

3.2.6. THE EFFECT OF TIME ON TREATMENT ON EPA+DHA IN HUMAN RBC AND HUMAN ATRIUM

The date of the commencement of treatment was available only for 15 subjects (FO n=8; placebo n=7). In blood samples (Figure 3.6, A-C), plotting days of treatment against the fatty acid concentration demonstrated the elevation of RBC EPA above baseline and above placebo, within approximately 15 days and was sustained thereafter. This was not so clearly identifiable in EPA+DHA or total n-3 PUFA because of the relatively lesser change in the major n-3 PUFA DHA with FO supplementation.

In atria (Figure 3.7, A-C), DHA appeared to be elevated above placebo treated concentrations only after 28 days of FO supplementation and this was similarly evident in plots of EPA+DHA and total n-3 PUFA. Similarly, the smaller increases in EPA were not evident before 28 days in atrial samples.

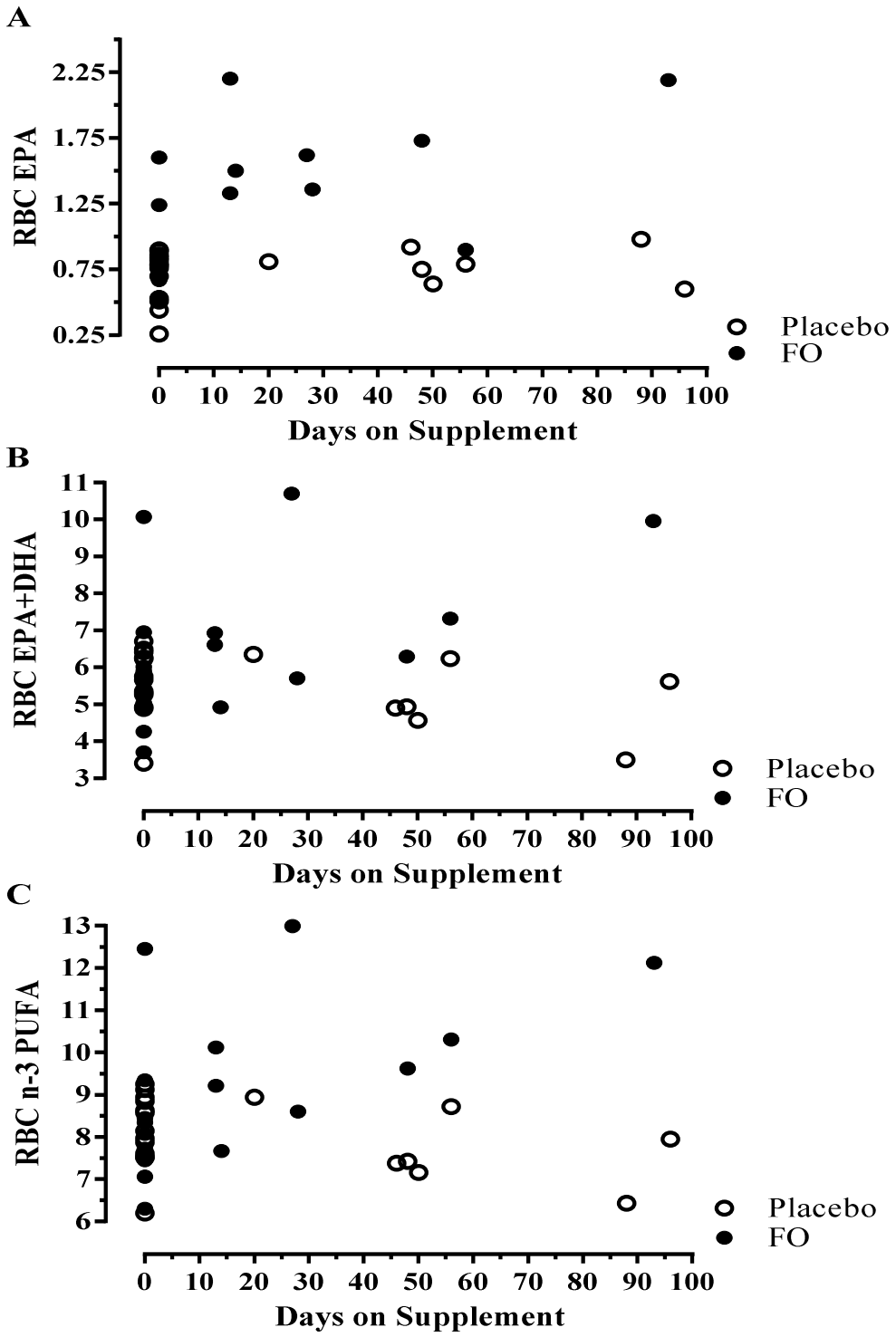

Figure 3.6. Dietary supplementation effect on RBC membrane polyunsaturated fatty acids over time.

A. Blood values of EPA maintain a higher concentration in FO subjects from approximately 15 days. **B.** RBC EPA+DHA is higher than placebo treated RBC after 28 days. **C.** Total RBC n-3 PUFA results are shifted ~2% upwards, generally by DPA.

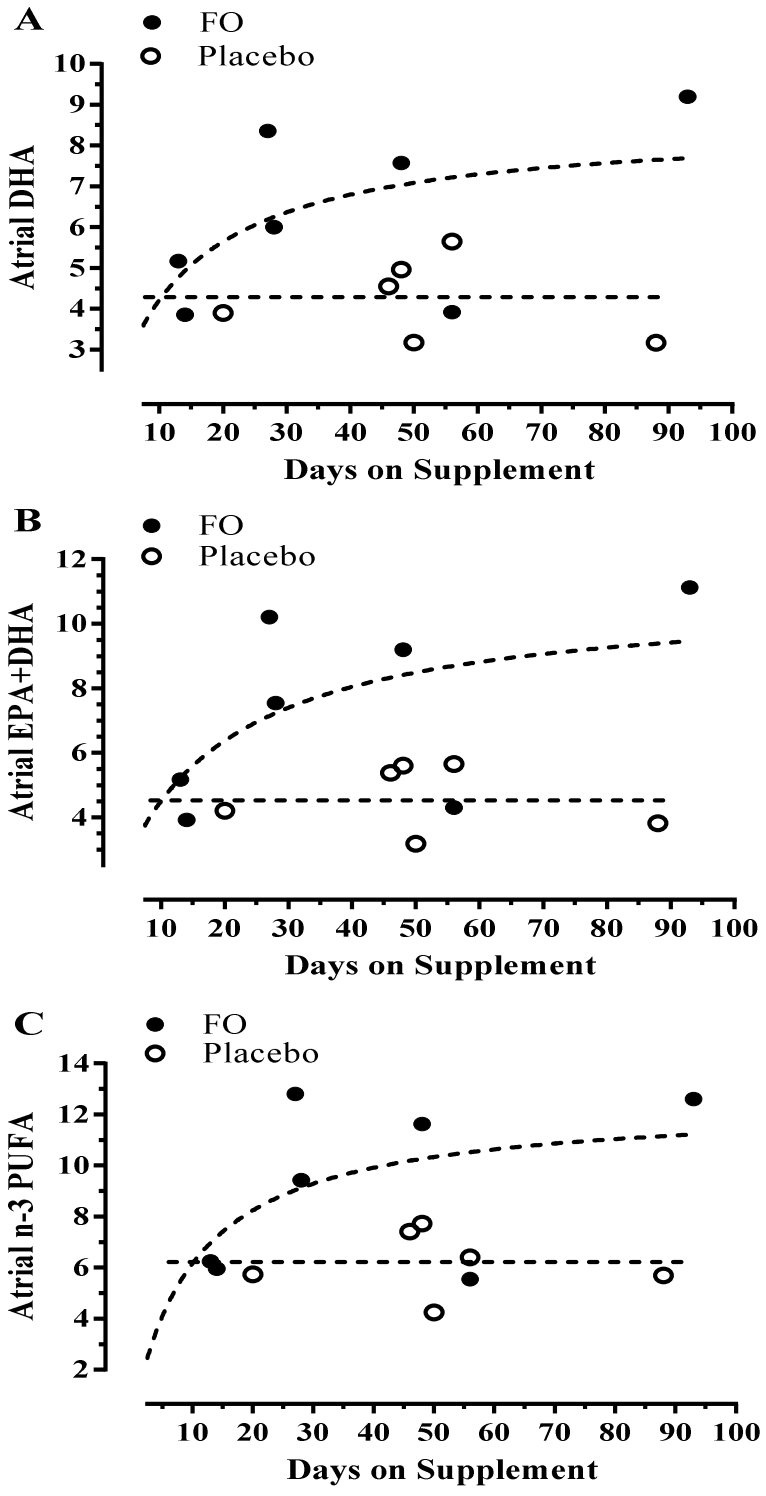

Figure 3.7. Fish oil dietary supplementation effect on atrial membrane polyunsaturated fatty acids over time.

A. Atria DHA nonlinear curve fit for FO, with linear fit for Placebo. **B.** Atria EPA+DHA nonlinear curve fit for FO. **C.** Atria n-3 PUFA nonlinear curve fit for FO.

3.3. DISCUSSION

The Omega-3 index (RBC EPA+DHA) has been proposed as a marker of cardiac risk (Harris*, et al.*, 2004a; Harris, 2009). Its use is based on the premise that the membrane fatty acid composition of red blood cells should represent cardiac n-3 PUFA content but can be more readily sampled. In turn, many of the cardiovascular protective effects of fish oil are likely related to physiological consequences of the incorporation of n-3 PUFA into cardiac tissue. This study showed that despite variations in concentrations of different PUFA in red blood cell membranes compared with atrial membranes and despite variations in PUFA responses to supplementation with fish oil, EPA+DHA in red blood cells provides the best representation of n-3 PUFA incorporation into atrial membranes.

The long term stability and reliability of red blood cell membrane fatty acid composition in unsupplemented subjects was demonstrated in two ways: first, as the absence of significant differences in any fatty acid concentration between the baseline samples of the placebo and FO treatment groups, and secondly as the absence of significant differences in any fatty acid between the baseline and peri-operative blood samples within the placebo group. On this basis we can be confident that any differences in fatty acid composition between the peri-operative blood sample and baseline in the FO treatment group are indeed due to the fish oil supplementation.

The composition of red blood cell samples from unsupplemented subjects was remarkably consistent with other published values from cardiac surgery candidates (Harris*, et al.*, 2004a; Garg*, et al.*, 2006; Metcalf*, et al.*, 2007). Saturated fatty acids made up 42% of the total, MUFA made up 18% of total and n-6 PUFA made up the majority of total PUFA. Arachidonic acid was in the greatest concentration for any

single PUFA and at 15% was about double the concentration of the main dietary essential fatty acid, LA (see Table 3.1). Amongst the n-3 PUFA, the main contributor was DHA, and there was similar consistency amongst published data with DHA present at almost double the concentration of its 22 carbon precursor DPA. The concentration of the 20 carbon EPA was lowest of the long chain n-3 PUFA, and ALA, the shorter plant sourced n-3 PUFA, was barely detectable. Metcalf and co-workers recently published a larger sample study (Metcalf*, et al.*, 2010), which is again consistent with the present results and previous publications. The consistency between this study and other studies reporting red blood cell fatty acids amongst cardiac surgery candidates within different regions of Australia (Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010) is particularly high and they contrast to the north American study of Harris (Harris*, et al.*, 2004a), which reported higher concentrations of n-6 PUFA LA (10% *c.f.* 7-8.5%) and AA (17% *c.f.* 14-14.5%) and lower omega-3 index (4.7% *c.f.* 5.1-6.3%). This may well represent differences in the usual dietary intakes of n-6 and n-3 PUFA between these populations.

Although fatty acid composition of individual tissues is highly reproducible and consistent from subject to subject, especially in animal studies with controlled diets, this contrasts with the variations in fatty acid composition according to tissue type seen both within animal species (Charnock*, et al.*, 1989; Bourre*, et al.*, 1997) and amongst human tissues (Arterburn*, et al.*, 2006). In the current study, the atrial samples contained greater concentrations of n-6 PUFA than were recorded in red blood cells, especially of LA, which was more than doubled and at 17% of total fatty acids was comparable to the AA concentration in atria. In contrast, the n-3 PUFA total and EPA+DHA were significantly lower than in the red blood cells, by virtue of significantly lower atrial EPA and DPA concentrations. These basic characteristics of atrial fatty acid

composition were in line with those previously published (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010) as were the key differences in n-6 and n-3 PUFA between atrial and red blood cell membrane fatty acid composition (Metcalf*, et al.*, 2007; Metcalf*, et al.*, 2010). The FO supplementation produced increases in EPA and DHA but not in DPA, which remained unchanged, as reported previously (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007). The largest changes in this and other studies were seen with the increase in atrial DHA (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007), despite vastly different supplementation rates and use of high EPA fish oils in previous studies.

With obvious inter-tissue differences consistently reported, the question to answer is: What is the best marker in red blood cell membrane to represent the n-3 PUFA composition of the heart? Does the Omega-3 index (using RBC EPA+DHA) provide the best marker of the composition of the human atrium? Whilst both EPA and DHA concentrations in blood cells correlated with their individual concentrations in atria, the stronger correlation and significant slope was seen in the correlation of EPA+DHA in blood and atria. Nothing was gained by evaluating individual n-3 PUFA nor did inclusion of DPA contribute either as a marker in the blood or as a marker of n-3 PUFA in the atria. In fact it impacted negatively in every consideration, with no correlation in DPA concentration between blood and atria (r^2 = 0.008) and poor correlation of EPA+DPA+DHA between blood and atria. This is reflected in the failure of fish oil supplementation to modify atrial DPA concentrations (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007) despite some increase in the red blood cells.

The n-3 PUFA, DPA (22:5 n-3) is an intermediate in the metabolism of n-3 PUFA from EPA to DHA. Whilst not commonly recorded as a component of fish oil supplements, DPA can be found in food sources such as red meat, and it has been proposed that this could be the main dietary source for many Australians and an alternative dietary source

of n-3 PUFA in those unwilling or unable to eat seafood (Howe*, et al.*, 2007). This then might suggest a potential role for DPA incorporation into cellular membranes as a marker of dietary intake or n-3 PUFA status. Indeed, although DPA was present in human red blood cells at almost half the DHA concentration, it reached 3-5 times the concentration of EPA, as previously reported (Harris*, et al.*, 2004a; Metcalf*, et al.*, 2007) and most recently (Metcalf*, et al.*, 2010). However, the concentration of DPA in red blood cells correlated very poorly with DPA in the atria or any other measure of n-3 status in the atria. In fact, red blood cell DPA had a significant negative correlation with red blood cell DHA and an even stronger negative correlation with atrial DHA in placebo subjects, which at $r^2 = 0.59$ ($p=0.002$) provided the best correlation of any found in this study. Notably, blood cell EPA was also negatively correlated with atrial DHA in placebo treated subjects. This suggests that in unsupplemented subjects there is some conversion of EPA and DPA to DHA or the turnover of previously acquired DHA is much slower. In their comprehensive assessment of 61 subjects Metcalf and co-workers (Metcalf*, et al.*, 2010) did not report these correlations, however their data shows that the correlations of red blood cell EPA and DPA with their respective atrial concentrations are less than unitary, indicating lower incorporation of EPA and DPA into atria than in red blood cells. This contrasts to DHA which is incorporated into atria at similar or greater concentrations, as was observed in the present study. When subjects were supplemented with fish oil, either high in DHA (this study and (Leong*, et al.*, 2010)) or high in both EPA and DHA (Garg*, et al.*, 2006; Metcalf*, et al.*, 2007) or higher in EPA (Harris*, et al.*, 2004a) there was very little or no change in DPA concentrations in blood or atria, in contrast to the changes in EPA and DHA.

Amongst these observations of variation in fatty acid concentrations and correlations, and despite some negative associations, the consistent finding of this study was that

EPA+DHA in red blood cell membranes is the best surrogate for n-3 PUFA status in atrial tissue, that holds up across the wide range of blood concentrations, whether subjects are naïve to fish oil or supplemented, confirming the recent report of Metcalf (Metcalf*, et al.*, 2010).

This study confirmed in human tissue as previously demonstrated in animals, that DHA is the principle n-3 PUFA in the membrane phospholipid fatty acids of both myocardium and RBC. Preferential incorporation of DHA into heart chamber membranes in rats is demonstrated by time- and dose-responses to FO supplementation (Owen*, et al.*, 2004; McLennan*, et al.*, 2007; Slee*, et al.*, 2010). This occurs regardless of a high background of n-6 PUFA in the diet (Slee*, et al.*, 2010), making it more relevant to Western human diets and gives validity to the results seen here. Recently, Metcalf (2010) was able to show that free-living individuals, who self-reported greater than two fish meals per week and some amount of FO supplementation (no high doses), had positive responses in DHA in the atrium and RBC membranes (see Table 3.1). It has also been shown that larger FO supplements, such as 4.4 *g*/day (Garg*, et al.*, 2006) or 6 *g*/day (Metcalf*, et al.*, 2007) will give larger increases in atrium membrane DHA and EPA over time. Further, in a random subset of Framingham Offspring subjects Harris (2012) was able to demonstrate an approximate 2% increase in RBC EPA+DHA from FO supplementation regardless of baseline RBC EPA+DHA (Harris*, et al.*, 2012). We are now able to more confidently transfer trends and conclusions from rodent data (myocardial membrane phospholipid fatty acids) to that of the human trends in myocardial membrane phospholipid fatty acid analyses. However, it can be argued of subjects with less than 42 days of FO supplementation, as seen in Garg (Garg*, et al.*, 2006), maximal incorporation of DHA into myocardial membranes has not been achieved and a limitation for analysis.

Herein, elective surgery for heart disease delivered a sizeable specimen of myocardial tissue to confirm an effect of oral FO supplementation on myocardial membrane phospholipid fatty acids. Earlier published membrane phospholipid fatty acid analyses of the human heart have presented data from cadaveric samples (Gudbjarnason, *et al.*, 1975a; Heckers, *et al.*, 1977; Gudbjarnason, *et al.*, 1978c; Belfrage, *et al.*, 1979; Ray, *et al.*, 1979; Sen, *et al.*, 1981; Sexton, *et al.*, 1995). Quasi-healthy intra-operative cardiac surgery samples have been obtained from various cardiac sites; including the atria (unknown side) (Garg, *et al.*, 2006), right-sided interventricular septum (Harris, *et al.*, 2004a), right atrial appendage (Metcalf, *et al.*, 2007), left ventricular myocardium as papillary muscle (Rocquelin, *et al.*, 1985) and endomyocardial biopsy (septal-apical region of right ventricle) (Belfrage, *et al.*, 1979). Animal studies however have marked differences in membrane FA composition from various sites within the heart (Charnock, *et al.*, 1983) and regional differences may account for some variation found in the reported values of phospholipid fatty acids found here and of those reports mentioned. Harris (2004) provided an early insight into the incorporation of omega-3 PUFA into human heart membranes in response to fish oil supplementation, unfortunately extremely low DHA concentrations and PUFA in general were reported, and on the basis of the current study and others must remain contentious, reflecting the possibility for the biopsy sample to be epithelium and not myocardium (Harris, *et al.*, 2004a).

The amount of myocardial tissue disruption in cardiac surgery for valve repair/replacement is very different to surgery for coronary artery bypass grafting (CABG), where CABG surgery can be portrayed intrinsically as vascular surgery. Damaged heart tissue or ischaemia, is determined by blood serum assay levels of troponin I (cTnI) (Adams, *et al.*, 1993); progressive ischaemia results in higher cTnI levels. Leong (2010) recently reported a reduction in cTnI levels in both CABG

(significantly) and valve surgery (non-significantly) after the inclusion of oral FO supplementation (3 g/day), as part of an oral metabolic therapy prior to surgery (Leong*, et al.*, 2010). Animal studies show post-ischaemic oxygen utilisation, cardiac output and external work are all improved after FO feeding (Pepe*, et al.*, 2002; McLennan*, et al.*, 2007; Pepe*, et al.*, 2007). Furthermore, FO incorporation limits myocardial infarct size, inhibits post-ischaemic arrhythmias and improves contractile recovery in ischaemia-reperfusion (Abdukeyum*, et al.*, 2008).

The delta-6 desaturase enzyme is the rate-limiting step in the bio-synthesis of the n-3 and n-6 PUFA long chain fatty acids, AA, EPA and DHA (Horrobin, 1993) (Chapter 1, Figure 1.1). With competition between substrate LA and ALA, precursor/product desaturation is reportedly preferentially directed in favour of n-3 PUFA (Brenner, 1974; Holman, 1986). However, dietary consumption is weighted more towards n-6 PUFA intakes, particularly LA (AHA*, et al.*, 2009), and conversion of ALA to EPA and DHA is limited in humans (Burdge*, et al.*, 2005; Arterburn*, et al.*, 2006). It is generally agreed that to overcome this imbalance, dietary consumption of preformed long chain fatty acids AA (from meat, eggs and some fish), EPA and particularly DHA (from fish or FO) is required. Investigation of n-6 and n-3 PUFA product/substrate ratios (Figure 3.1) in this study demonstrated the common trend in red blood cell membrane and myocardial membrane phospholipid fatty acids, and in the face of FO supplementation. Compared to atria, red blood cell membranes showed higher ratios of arachidonic (20:4n-6) to linoleic acid (18:2n-6) (AA/LA) and 22:4n-6 to arachidonic acid (22:4/AA) within the n-6 PUFA, and they remained relatively constant between placebo and FO supplemented subjects. The atria also had higher ratios of the n-3 PUFA, docosahexaenoic (22:6n-3) to docosapentaenoic acid (22:5n-3) (DHA/DPA), which was further increased with FO supplementation.

Cardiac surgery patients are at a greater risk for post-operative cardiac arrhythmias, such as atrial fibrillation (AF) after CABG and valve repair (Ommen, *et al.*, 1997). Several human trials have shown AF to be reduced post-operatively after FO supplementation (Calò, *et al.*, 2005; Leong, *et al.*, 2010); Leong used fish oil in conjunction with other oral metabolic supplements which reduces certainty that the observed effects can be specifically attributed to the fish oil, moreover three randomised controlled trials (Saravanan, *et al.*, 2009; Heidarsdottir, *et al.*, 2010; Farquharson, *et al.*, 2011) have recently found no effect on AF. This is an intriguing area of requiring further research. Also, fish consumption as tuna and baked or broiled fish from one to greater than five serves each week was associated with a 28% to 31% reduction in risk of AF in the elderly (Mozaffarian, *et al.*, 2004). Initially evidence from animal studies demonstrated the strong influence of reduced ventricular arrhythmias following a diet which included FO (McLennan, *et al.*, 1988; McLennan, 2001), especially in cross-over diets of saturated fat to FO (McLennan, *et al.*, 1990; Pepe, *et al.*, 2007). However in contrast to ventricular arrhythmias, no animal models exist that can demonstrated experientially the efficacy in preventing AF and clinically, some contention still remains regarding the cardiac benefits of FO and the incidence of AF from observational studies (London, *et al.*, 2007) and one large prospective trial (Kowey, *et al.*, 2010) in non-surgical patients. When fish oil supplements are initiated only at pre-surgical check, which is usually planned no more than 2 weeks later, the effectiveness of the intervention, requiring incorporation into membranes of the atria may also be obscured by insufficient time for adequate incorporation.

Based on the basic effects of n-3 PUFA EPA on eicosanoid metabolism and platelet aggregation, there has been a long-held concern that pre-treating patients with fish oil could put them at risk of bleeding events. This is particularly important in

cardiovascular disease patients who may need surgery to correct valves or coronary vascular stenosis. Blood loss is a major concern for any cardiac surgery, peri- or post-operatively and there is some evidence to suggest an increased risk of adverse outcome after RBC transfusions (Murphy*, et al.*, 2007). However, length of stays in hospital for surgical procedures vary widely and peri-operative measures with supplementation of omega-3 fatty acids can be seen to assist patients in reducing hospital stays by 1 - 2 days and thus, hospital costs (Leong*, et al.*, 2010). Importantly, a period of high FO supplementation prior to cardiac surgery was shown not to increase specifically measured blood loss peri- or post-operatively (Metcalf*, et al.*, 2007).

There have been volumes written on various mechanisms displaying attributes towards the initiation and progression of heart disease in animals and humans. Alternatively, under the influence of the omega-3 PUFA some mechanisms can inhibit heart disease progression (McLennan*, et al.*, 1996; Stillwell*, et al.*, 2003) with effects on inflammation, thrombus formation, vascular endothelium, smooth muscle cells, blood triacylglycerides and cell membrane components (Adkins*, et al.*, 2010) and intracellular enzymes and cell signalling (Siddiqui*, et al.*, 2008).

This study chapter was able to demonstrate the utility of the Omega-3 Index using data derived from red blood cell membrane and human atrium membrane phospholipid fatty acids taken from the same human individuals. Notwithstanding variations demonstrated in PUFA concentrations of red blood cell and atrium samples and regardless of variations seen in PUFA responses after fish oil supplementation, EPA+DHA in red blood cells is shown to be the best demonstration of n-3 PUFA incorporation into atrial membranes.

3.4. CONCLUSION

This study clearly shows that the composition of membrane phospholipid fatty acids of human atria can be changed through dietary means by the consumption of omega-3 fatty acids as FO. We can only show this incorporation if we have baseline blood samples and post-supplementation blood samples. The response would be the same for atrial samples also, if the technique of heart biopsy was neither invasive nor dangerous.

These results clearly demonstrate the Omega-3 Index positively indicating a correlation to human atrium EPA+DHA content. From this study the 'Omega-3 Index' can be used as a marker of right atrial membrane EPA+DHA established by direct comparison of human atrial membrane and RBC membrane samples.

Differences in membrane fatty acid compositions of different tissues in humans as shown here reflect those of compositional differences seen in animals, especially in the heart and red blood cell membranes and as such, support the role for continuing animal studies in the area of dietary modification and membrane analyses.

This study established that DHA is the main n-3 PUFA in human heart and atrial biopsy.

Chapter 4

OMEGA-3 FATTY ACIDS IN THE HUMAN HEART: CORRELATION BETWEEN ATRIA, VENTRICLE AND ERYTHROCYTES

Discussion and Final Conclusions

4.0. INTRODUCTION

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are cardioprotective in man and in animals. Controlled animal studies indicate that, independently of effects on vasculature, platelets and blood lipids, which may influence blood pressure, atherosclerosis and other inflammatory processes, many cardioprotective effects are associated with the incorporation of the n-3PUFA into myocardial membranes. The percentage of eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA) in the human red blood cell (RBC) (termed the Omega-3 Index) correlates inversely with adverse cardiovascular outcomes and is proposed as a cardiovascular risk factor (Harris & von Schacky, 2004). There are no known influences on red blood cell properties that could directly contribute to this reduced risk. Therefore the association is made on the premise that RBC membrane is a marker for n-3 PUFA availability for action or incorporation at an alternative site, specifically the heart and therefore reflects the composition of myocardium EPA+DHA concentrations. In particular it should reflect the incorporation into ventricular myocardium if it is to indicate the risk of fatal ventricular arrhythmias, myocardial dysfunction or heart failure.

Patterns of incorporation of DHA, as the predominant n-3 PUFA in membrane phospholipid fatty acids (Arterburn*, et al.*, 2006), and other fatty acids, vary across different tissue types, such as the heart, brain and RBC, in humans (Arterburn*, et al.*, 2006), just as they do in animals. This was confirmed in Chapter 3, where compositional differences between atria and RBC were observed within subjects when sampled at the same time, with or without fish oil supplementation, that were not seen between subjects in RBC samples taken at different times, unless subject to fish oil

intervention. Clearly the compositional differences between atria and RBC are real differences, not due to sampling error or inter-subject variability.

Myocardial samples for membrane phospholipid fatty acid analysis are usually taken opportunistically from living humans during cardiac surgery or in routine post-operative heart transplant checks. The quality of some human atrial or ventricular biopsy sampling can be questioned, as for example, previous reports have shown lower than expected DHA and EPA concentrations in fresh ventricle samples when compared to other studies from cadaveric or fresh peri-surgical sampling (Chapter 3, Table 3.1). With respect to n-3 PUFA in the present study EPA and DPA concentrations in atria were significantly lower than RBC, but no difference was seen in DHA. Nevertheless, these membrane phospholipid fatty acids in the heart (EPA+DHA) showed the best correlation with the more readily obtained RBC membrane phospholipid EPA+DHA (Chapter 3, Table 3.4) for Omega-3 Index assessment.

It was unclear however if the above measures could be reflective of membrane phospholipid EPA+DHA of the left ventricle (as the prime functional focus of cardiac risk). It was the purpose of this thesis to set a baseline for human ventricular fatty acid composition and establish the relationship between EPA+DHA in human RBC, atria and ventricles.

4.1. EPA AND DHA IN HUMAN RED BLOOD CELLS AND THE HUMAN HEART

This thesis has established that docosahexaenoic acid is the major n-3 PUFA in membrane phospholipid fatty acid in human heart (both atria and ventricle) and in RBC.

This preferential incorporation of DHA over EPA was further emphasised when FO supplementation (Chapter 3) provided EPA > DHA but DHA concentration increased more than EPA concentration did in both RBC and atria. Although significant differences between donor right atria DHA and donor left ventricle DHA were identified (Chapter 2, Table 2.3), EPA concentration was less than 1% of the total fatty acids in these tissues and no significant differences in EPA concentrations were identified between the tissues. The increase in DHA concentration after FO supplementation was also greater in atria than in RBC and the concentration range (particularly upper limits) in heart tissue (especially atria) was greater than in RBC; DHA ranges: right atria biopsy of 3.2 - 7.6 % (% total fatty acids), donor right atria of 2.9 - 10.8 %, donor left ventricle of 2.5 - 6.6% and red blood cells DHA of 3.5 - 6.3 % (means±SD are shown in Chapter 2 and Chapter 3, Figure 3.2 & 3.3). This variability in control (unsupplemented) samples suggests that the myocardial DHA concentration may be highly susceptible to dietary availability (provision or deficiency).

4.1. Table-1. Important n-3 PUFA content of several human tissue types without fish oil supplementation.

	EPA	*DHA	^EPA+DHA
RBC (n=13)	0.69±0.23	[a b]5.11±0.78	[a b]5.65±0.77
Right Atria Biopsy (n=14)	0.28±0.09	[a b]4.54±1.12	[a b]4.82±1.10
Donor Right Atria (n=16)	0.74±0.30	[a] 6.12±2.1	[a] 6.86±2.33
Donor Left Ventricle (n=16)	0.75±0.39	[b] 4.42±1.23	[b] 5.17±1.56

Results are mean±SD% of total fatty acids; *****DHA - a General ANOVA identified significant differences in tissues, p=0.0217 with Tukey HSD multiple comparisons, p<0.05, identifying [a]Right Atria Donor significantly different to [b]Left Ventricle Donor; ^EPA+DHA - a General ANOVA identified a trend towards a significant differences in tissues, p=0.0608 with Tukey HSD multiple comparisons, p< 0.05, identifying Donor Right Atria as significantly different to Donor Left Ventricle.

Neither mean DHA nor mean EPA+DHA concentration in RBC was significantly different to mean myocardial concentrations in atria or ventricle in the absence of fish oil supplementation (Table 4.1).

4.2. TISSUE FATTY ACID CORRELATIONS IN HUMAN SUBJECTS

The Omega-3 Index (red blood cell EPA+DHA concentration), correlated within human subjects to their matched right atrial membrane phospholipid EPA+DHA concentration ($p=0.0004$) (Figure 3.4); with a close to unitary correspondence to concentrations between the two tissues. A similar finding was shown by Metcalf (Metcalf*, et al.*, 2010) from a larger sample size. Moreover, Metcalf (Metcalf*, et al.*, 2010) and the present study both concur in showing a unitary correspondence between RBC and right atrial DHA yet a less than unitary correspondence between RBC EPA and right atrial EPA (RBC EPA > right atrial EPA).

The missing link in confirming the direct association between the RBC Omega-3 Index to cardiac risk is its correspondence to ventricular tissue concentration. Ventricular muscle samples are rarely obtained under ideal conditions (e.g. cadaveric samples, see Chapter 2). Those that have been obtained as intraventricular biopsy, despite their correlation with RBC omega-3 index and dose response to fish oil supplementation (Harris*, et al.*, 2004a) (Table 1.1) most likely represent the composition of ventricular endothelium, being so very different in composition to either fresh ventricular samples of this and other studies (papillary biopsy: (Rocquelin*, et al.*, 1985) or cadaveric ventricular samples (Gudbjarnason*, et al.*, 1978b; Belfrage*, et al.*, 1979; Sexton*, et al.*,

1995); low in both n-6 PUFA and n-3 PUFA and more in line with composition of human buccal epithelial (cheek) cells (Arterburn*, et al.*, 2006).

Matched samples from human donor right atria and donor left atria were strongly correlated in membrane phospholipid EPA+DHA concentrations in donor subjects (Figure 4.1). As with the correlations between RBC and right atrium, the relationship while strongly correlated, was not unitary, with higher concentrations of EPA+DHA in right atria than in left atria.

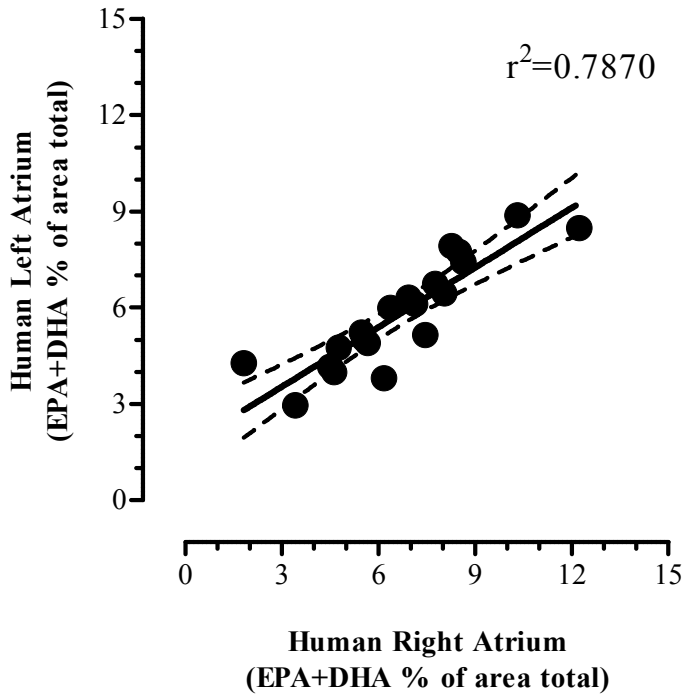

Figure 4.1. Membrane EPA+DHA in donor right atrial samples and donor left atrial samples from heart transplant programme.

Linear regression line with 95% CI (confidence interval) for right atrium EPA+DHA goodness of fit to left atrium EPA+DHA (n=20), $r^2 = 0.7870$. The slope is significantly non-zero $p<0.0001$. Data from Chapter 2

Matched samples from human donor left atria and donor left ventricles were also strongly correlated in membrane phospholipid EPA+DHA concentrations in donor subjects (r^2=0.8, non-zero slope significance p<0.0001, n=20) (Figure 4.2) but unlike the left and right atria showed one: one or unitary correspondence between the tissues.

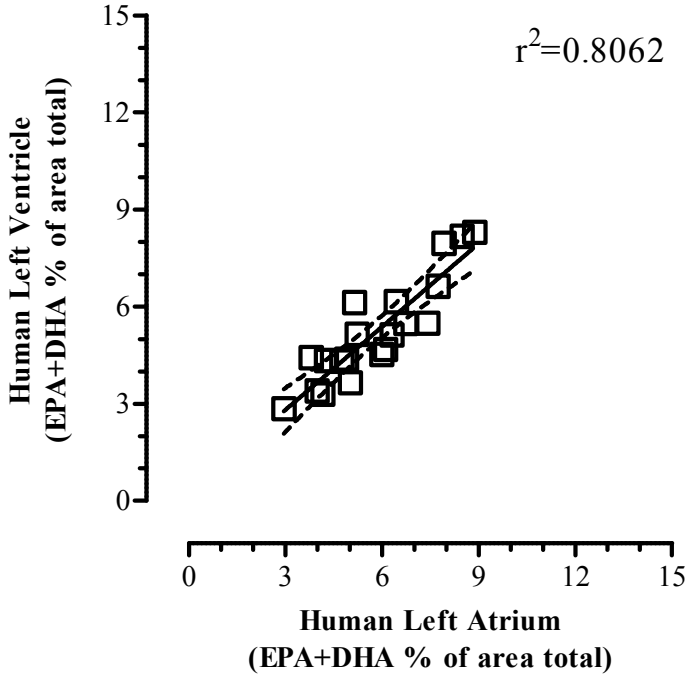

Figure 4.2. Membrane EPA+DHA content of human left atrium and left ventricle in donor hearts from transplant programme.

Linear regression line with 95% CI (confidence interval) for red blood cell EPA+DHA goodness of fit to right atrium EPA+DHA (n=20), r^2 = 0.8062. The slope is significantly non-zero *p*<0.0001. Data from Chapter 2

Matched samples from human donor right atria and donor left ventricles were strongly correlated in membrane phospholipid EPA+DHA concentrations in donor subjects (r^2=0.7, non-zero slope significance p<0.0001, n=20) (Figure 4.3) and like the left and

right atria showed non-unitary correspondence between tissues, with right atrial concentrations higher than corresponding left ventricle.

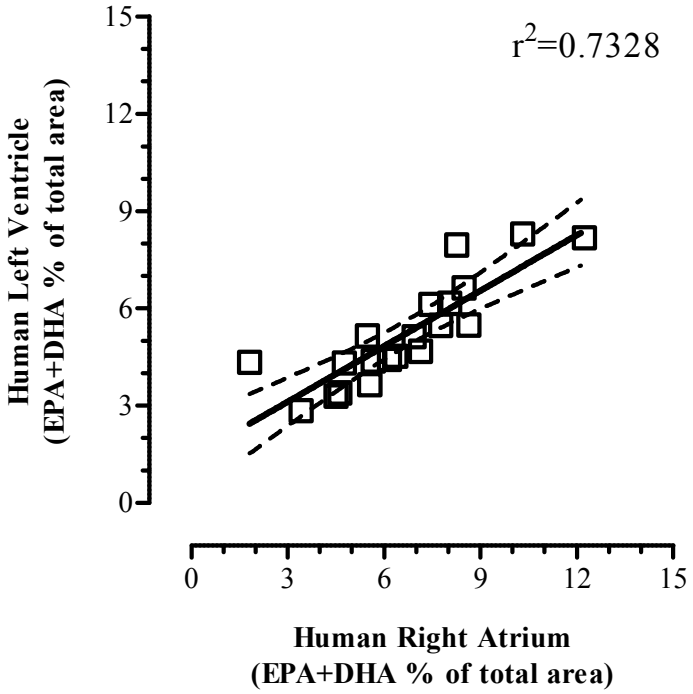

Figure 4.3. Membrane EPA+DHA in human right atrium and left ventricles in donor.

Linear regression line with 95% CI (confidence interval) for red blood cell EPA+DHA goodness of fit to right atrium EPA+DHA (n=20), $r^2 = 0.7328$. The slope is significantly non-zero $p<0.0001$. Data from Chapter 2

4.3. DISCUSSION

Medical science has shown that n-3 PUFA are cardioprotective in numerous animal models such as dogs (Billman*, et al.*, 1994; Billman*, et al.*, 1999), cats (Reibel*, et al.*,

1986), rats (McLennan, 2001; McLennan, *et al.*, 1988; Pepe & McLennan, 1996, 2002), rabbits (Murnaghan, 1981; Den Ruijter, *et al.*, 2012) and mice (Huggins, *et al.*, 2009). Docosahexaenoic acid has been identified as the principal n-3 PUFA in rat myocardium (Gudbjarnason, *et al.*, 1975a; Charnock, *et al.*, 1983) and in other animals such as the non-human primate, marmoset monkey (Charnock, *et al.*, 1985a), mouse (Huggins, *et al.*, 2009), cat (Reibel, *et al.*, 1986) and dog (Billman, *et al.*, 2011) and it is the purported active constituent of fish oil in animal studies (McLennan et al. 1996). However, the often extremely high myocardial concentrations of DHA in many animals, particularly in small rodents after fish oil supplementation, raises a question over the relevance of animal studies to the human. Nevertheless, this thesis, through a series of analyses using human RBC, atrial biopsy and transplant donor hearts has confidently established that DHA is the major human heart n-3 PUFA and that the Omega-3 Index (Harris, *et al.*, 2004b) can be used as a valid marker of human heart EPA+DHA composition. Moreover, the range of human heart DHA concentrations overlaps with unsupplemented rat heart concentrations and follows on from the demonstration that only small supplemental intakes in the rat, equivalent to achievable human dietary intakes are required to modulate the rat myocardium (Slee, *et al.*, 2010). For the first time, individual chambers of the human heart have been evaluated to highlight the strong correlations between myocardium EPA+DHA right atrium and left atrium, left atrium and left ventricle, and right atrium versus left ventricle. These correlations all point towards the validity of the RBC derived Omega-3 Index as a marker for EPA+DHA content of the human heart.

The Omega-3 Index has been identified by several studies to give close associations between RBC EPA+DHA and myocardial membrane EPA+DHA (Harris, *et al.*, 2004a; Metcalf, *et al.*, 2007; Metcalf, *et al.*, 2010), the analyses here show that myocardial

membrane EPA+DHA is also strongly correlated between various chambers of the heart. Harris makes a strong case for a low Omega-3 Index to be considered as a risk factor for cardiovascular disease (Harris, 2007) along with high cholesterol, hypertension, obesity and diabetes This marker can be used as a relatively non-invasive tool, and could be utilized along with current diagnostic tools by clinicians in determining appropriate preventative strategies for the support of heart health in our communities, where coronary heart disease was the leading cause of death in 2007 in Australia (NHFA, 2006; AIHW, 2010); future healthcare expenditure is projected to cost more than $1 billion per annum in Australia due to heart failure and an ageing population alone (Clark*, et al.*, 2004).

As demonstrated in this thesis, the stress of advancing age in humans (see Chapter 2) corresponded to higher DHA concentrations in myocardial membranes of donor and explanted failing hearts increasing DHA concentrations in the heart also correspond to the stress of cardiac failure. Similarly, myocardial DHA is seen to increase under stress conditions in animals (Gudbjarnason*, et al.*, 1975b) as well as in animals with faster heart rates and higher metabolic rates (Gudbjarnason*, et al.*, 1978c; Pepe*, et al.*, 2002). It even increases in the rat in association with saturated fat feeding (relative to a control or n-6 PUFA diet (Charnock*, et al.*, 1985b; Pepe*, et al.*, 1996) and its associated increases in heart rate and oxygen consumption (Pepe*, et al.*, 2002, 2007). Myocardial DHA is also increased by dietary supplementation with fish oil in both animals (Gudbjarnason*, et al.*, 1977; Charnock, 1985; Abeywardena*, et al.*, 1987) and in man (Harris*, et al.*, 2004a; Metcalf*, et al.*, 2007) and as observed in this thesis. These make contrasting observations, with myocardial DHA being elevated in response to stress or in animals with high heart rates and metabolic rates, yet dietary incorporation of n-3 PUFA in man and animals being associated with reduced heart rate and oxygen

consumption, and improved myocardial function and associated cardiovascular risk outcomes. Therefore, this thesis supports the premise that stress induced increases in DHA are somehow physiologically adaptive (McLennan*, et al.*, 2005) rather than causative of cardiac dysfunction. There are no previous studies of membrane phospholipid fatty acids from viable, healthy left ventricular muscle in humans (see Chapter 2), except post-transplant biopsy samples from the right interventricular septum (Harris*, et al.*, 2004a). Interestingly, the reports of the DHA concentrations found within the left papillary muscle in chronic rheumatic heart disease (Rocquelin*, et al.*, 1985) are comparable to those found here.

It is commonly acknowledged that the diet influences the composition of cell membrane phospholipid fatty acids in most tissues in humans (Arterburn*, et al.*, 2006). Throughout this study the wide range of tissue EPA+DHA results indicate wide variations seen in dietary regimes found within the general community from the same relative geographical region of Melbourne (Australia), but as established in previous studies, the principle n-3 PUFA identified in each chamber of human donor heart membrane phospholipids is DHA, with EPA playing a minor role. There is continued conjecture as to the minimum and maximum levels of EPA and DHA to be consumed in the diet for achievable and adequate tissue levels for heart protection (Harris*, et al.*, 2009; Kris-Etherton*, et al.*, 2009; Lee*, et al.*, 2008).

4.4. CONCLUSIONS

This thesis has established that DHA is the main n-3 PUFA in human heart as it is in animal studies, with a range that overlaps with unsupplemented and low-dose

supplemented laboratory rat. The DHA concentration is elevated as an apparently compensatory response to stressors and in response to dietary fish oil, as it is in animal studies. With DHA commonly the main n-3 PUFA of table fish, but EPA the principle n-3 PUFA provided as supplements in many clinical trials, establishing the pre-eminent position of DHA amongst n-3 PUFA in human myocardium increases confidence in the consistent human epidemiological studies associating usual fish consumption with cardiovascular outcomes and in translating outcomes of animal studies to interpret mechanisms of n-3 PUFA action in man. It also may provide some explanation for the more variable outcomes of clinical trials.

Despite the consistent predominance of DHA over EPA and variations in their relative concentrations in myocardium and RBC, this thesis has established that the omega-3 index provides the most robust correlations of red blood cells with human heart tissue and can be regarded as a good indicator of myocardial membrane n-3 PUFA composition, confirming its potential as a marker of cardiac-associated risk.

4.5. IMPLICATIONS

Human dietary intake is most often predominantly DHA (Arterburn*, et al.*, 2006), due to the common predominance of DHA in table fish (Kris-Etherton*, et al.*, 2000; Services, 2002; U.S. Department of Agriculture, 2009). However, commercial fish oil supplements (such as menhaden oil) are most commonly rich in EPA and low in DHA. In an extreme example, the JELIS study (Yokoyama*, et al.*, 2007) which failed to reduce risk of sudden death (direct cardiac effect) whilst reducing risk of new events (coronary vascular effect) in patients post-MI, used a pure EPA supplement, in contrast

to the GISSI-P study which prevented sudden death but not new events in a similar clinical population using a high concentration supplement containing almost 400mg DHA.

The present study also raises some issues relating to contradictory animal studies, which against the backdrop of a high volume of experimental studies showing antiarrhythmic effects of dietary fish oil found pro-arrhythmic effects of extremely high intakes of fish oil in the dog (Billman*, et al.*, 2012). This recent study (Billman*, et al.*, 2012), which found that 4g/day fish oil in the dog (equivalent to 16g/day fish oil in a 20kg man), also revealed that the dog is a very poor incorporator of omega-3 DHA into myocardium. With a mean DHA incorporation in unsupplemented dogs (% DHA in: RBC 0.25; right atrium 0.38; left ventricle 0.36) well below the range observed in human hearts in this thesis, even after 4g fish oil per day supplementation DHA incorporation remained at the very bottom of the ranges observed in human hearts (% DHA in: RBC 2.83; right atrium 2.71; left ventricle 2.99). Even though the rat heart can incorporate extraordinarily high percentage of fatty acids as DHA with high fish oil supplements, the current study has established that DHA incorporation into human heart falls into the range of concentrations seen in hearts of unsupplemented rats or rats supplemented in the low dose range equivalent to human intakes of 1-2 fish meals per week (Slee*, et al.*, 2010).

4.6. FUTURE DIRECTIONS

This project has resulted in a good body of results for the normal human heart, and has given a view of failing myocardial phospholipid fatty acid composition. The establishment of DHA as the principal n-3 PUFA in human heart, even after dietary

supplementation with a predominantly EPA fish oil, may have implications for the planning of future intervention studies and for interpretation of clinical studies, which somewhat controversially do not always replicate the consistent human observational studies related to usual diet.

A major limitation to this study is the lack of dietary information and fish oil supplementation available for both heart transplant recipients and donor hearts. Donor hearts are assumed to be influenced by normal habitual diets, but it cannot be ruled out that heart transplant recipients have not taken oral fish oil supplements or increased their intakes of fish in their diets; with females more likely to be taking FO supplements. We can see however that prior cadaveric study have not included this information and evidence from other heart stress conditions, such as that from Benediktsdottir et al. (1988) and Gudbjarnason et al. (1975b), show our membrane phospholipid fatty acid information follows similar patterns.

The studies presented here show limited numbers of failing (explanted) (<14%) and donor hearts from female participants (<25%), which is a limitation to fully elucidate the gender description of myocardial membrane phospholipid fatty acids. As observed from numerous studies in this area, female participation is shown to be approximately 25% or less of total number of subjects which corresponds to data presented here.

References

Aarsetoey, H., Aarsetoey, R., Lindner, T., Staines, H., Harris, W.S., & Nilsen, D.W.T. (2011). Low levels of the omega-3 index are associated with sudden cardiac arrest and remain stable in survivors in the subacute phase. *Lipids, 46*, 151-161. doi: 10.1007/s11745-010-3511-3

Abdukeyum, G.G., Owen, A.J., & McLennan, P.L. (2008). Dietary (n-3) long-chain polyunsaturated fatty aicds inhibit ischaemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischeamic preconditioning. *The Journal of Nutrition, 138*(10), 1902-1909.

Abedin, L., Lien, E.L., Vingrys, A.J., & Sinclair, A.J. (1999). The effects of dietary ALA compared to DHA on brain, retina, liver, and heart in the guinea pig. *Lipids, 34*(5), 475–482.

Abete, P., Napoli, C., Santoro, G., Ferrara, N., Tritto, I., Chiariello, M., . . . Ambrosio, G. (1999). Age-related decrease in cardiac tolerance to oxidative stress. [Proceedings Paper]. *Journal of Molecular and Cellular Cardiology, 31*(1), 227-236.

Abeywardena, M.Y., McLennan, P.L., & Charnock, J.S. (1987). Long-term saturated fat feeding induced changes in rat myocardial phospholipid fatty acids and are reversed by cross-over to polyunsaturated diets: Differences between n-3 and n-6 lipid supplements. *Nutrition Research, 7*, 743-753.

ACC/AHA, Hunt, S.A., Baker, D.W., Chin, M.H., Cinquegrani, M.P., Feldman, A.M., . . . Stevenson, L.W. (2001). ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Revise the 1995 Guidelines for the Evaluation and Management of Heart Failure). *Journal of the American College of Cardiology, 38*(7), 2101-2113.

Adams, J.E.d., Bodor, G.S., Davila-Roman, V.G., Delmez, J.A., Apple, F.S., Ladenson, J.H., & Jaffe, A.S. (1993). Cardiac troponin I. A marker with high specificity for cardiac injury. *Circulation, 88*(1), 101-106.

Adkins, Y., & Kelley, D.S. (2010). Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *Journal of Nutritional Biochemistry, 21*(2010), 2010. doi: 10.1016/j.jnutbio.2009.12.004

AHA, Harris, W.S., Mozaffarian, D., Rimm, E.B., Kris-Etherton, P.M., Rudel, L.L., . . . Sacks, F. (2009). Omega-6 fatty acids and risk for cardiovascular disease: A science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. *Circulation, 119*(6), 902-907.

AHA, Kris-Etherton, P.M., Harris, W.S., & Appel, L.J. (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease: A Scientific Statement by the American Heart Association Science Advisory and Coordinating Committee. *Circulation, 106*, 2747-2757. doi: 10.1161/01.CIR.0000038493.65177.94

AHA, Lichtenstein, A.H., Appel, L.J., Brands, M., Carnethon, M., Daniels, S., . . . Wylie-Rosett, J. (2006). Diet and Lifestyle Recommendations Revision 2006. A Scientific Statement from the American Heart Association Nutrition Committee. *Circulation, 114*, 82-96.

AIHW. (2010). *Australia's Health 2010* (Vol. 12). Canberra: Australian Institute of Health and Welfare.

al Makdessi, S., Brandle, M., Ehrt, M., Sweidan, H., & Jacob, R. (1995). Myocardial protection by ischemic preconditioning: the influence of the composition of myocardial phospholipids. *Molecular and cellular biochemistry, 145*(1), 69-73.

Albert, C.M., Campos, H., Stampfer, M.J., Ridker, P.M., Manson, J.E., Willett, W.C., & Ma, J. (2002). Blood levels of long-chain n–3 fatty acids and the risk of sudden death. *New England Journal of Medicine, 346*(15), 1113-1118.

Albert, C.M., Chae, C.U., Grodstein, F., Rose, L.M., Rexrode, K.M., Ruskin, J.N., . . . Manson, J.E. (2003). Prospective study of sudden cardiac death among women in the United States. *Circulation, 107*(16), 2096-2101.

Albert, C.M., Hennekens, C.H., O'Donnell, C.J., Ajani, U.A., Carey, V.J., Willett, W.C., . . . Manson, J.E. (1998). Fish consumption and risk of sudden cardiac death. *Journal of the American Medical Association, 279*(1), 23-28.

Arterburn, L.M., Hall, E.B., & Oken, H. (2006). Distribution, interconversion, and dose response of n-3 fatty acids in humans. *American Journal of Clinical Nutrition, 83*(6), S1467-1476.

Ascherio, A., Rimm, E.B., Stampfer, M.J., Giovannucci, E.L., & Willett, W.C. (1995). Dietary intake of marine n-3 fatty acids, fish intake, and the risk of coronary disease among men. *New England Journal of Medicine, 332*(15), 977-982.

Bang, H.O., & Dyerberg, J. (1972). Plasma lipids and lipoproteins in Greenlandic West Coast Eskimos. *Acta Medica Scandinavica, 192*(1-2), 85-94.

Bang, H.O., & Dyerberg, J. (1980). Lipid metabolism and ischemic heart disease in Greenland Eskimos. *Advances in Nutritional Research (3), Chapter 1*, 1-22.

Bang, H.O., Dyerberg, J., & Hjorne, N. (1976). The composition of food consumed by Greenland Eskimos. *Acta Medica Scandinavia, 200*((1-6)), 69-73.

Bang, H.O., Dyerberg, J., & Nielsen, A.B. (1971). Plasma lipid and lipoprotein pattern in Greenlandic west-coast eskimos. *The Lancet*, 143-146.

Barceló-Coblijn, G., & Murphy, E.J. (2009). α-linolenic acid and its conversion to longer chain n-3 fatty acids: Benefits for human health and a role in maintaining tissue n-3 fatty acid levels. *Progress in Lipid Research, 48*, 355-374.

Baur, L.A., O'Connor, J., Pan, D.A., Wu, B.J., O'Connor, M.J., & Storlien, L.H. (2000). Relationships between the fatty acid composition of muscle and erythrocyte membrane phospholipid in young children and the effect of type of infant feeding. *Lipids., 35*(1), 77-82.

Belfrage, P., Johansson, B.W., Torp, A., & Akesson, B. (1979). Micromethods for anaIysis of lipids in endomyocardial biopsy specimens. *Acta Medica Scandinavica, 205*(4), 283-286.

Benediktsdottir, V.E., & Gudbjarnason, S. (1986). Arachidonic and docosahexaenoic acid content of sarcolemmal phospholipids in relation to ventricular fibrillation in rats. *Journal of Molecular and Cellular Cardiology, 18*(Supplement 2), 90.

Benediktsdottir, V.E., & Gudbjarnason, S. (1988). Reversible alterations in fatty acid composition of heart muscle membrane phospholipids induced by epinephrine in rats fed different fats. *Journal of Lipid Research, 29*(6), 765-772.

Bernard, M., Gerbi, A., Barbey, O., Jamme, I., Cozzone, P.J., & Maixent, J.M. (1999). Dietary fish oil promotes positive inotropy and efficiency of digitalis. *Lipids, 34*(Suppl), S195.

Bertrand, L., Horman, S., Beauloye, C., & Vanoverschelde, J.L. (2008). Insulin signalling in the heart. [Review]. *Cardiovascular Research, 79*(2), 238-248.

Besse, S., Delcayre, C., Chevalier, B., Hardouin, S., Heymes, C., Bourgeois, F., . . . Swynghedauw, B. (1994). Is the Senescent Heart Overloaded and Already Failing. *Cardiovascular Drugs and Therapy, 8*(4), 581-587.

Billman, G.E., Carnes, C.A., Adamson, P.B., Vanoli, E., & Schwartz, P.J. (2012). Dietary omega-3 fatty acids and susceptibility to ventricular fibrillation: lack of protection and a proarrhythmic effect. *Circulation. Arrhythmia and electrophysiology, 5*(3), 553-560. doi: 10.1161/circep.111.966739

Billman, G.E., Hallaq, H., & Leaf, A. (1994). Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proceedings of the National Academy of Sciences of the United States of America, 91*(10), 4427-4430.

Billman, G.E., & Harris, W.S. (2011). Effect of dietary omega-3 fatty acids on the heart rate and the heart rate variability responses to myocardial ischemia or submaximal exercise. *American Journal of Physiology - Heart and Circulatory Physiology, 300*(6), H2288-H2299. doi: 10.1152/ajpheart.00140.2011

Billman, G.E., Kang, J.X., & Leaf, A. (1999). Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. *Circulation, 99*(18), 2452-2457.

Billman, G.E., Nishijima, Y., Belevych, A.E., Terentyev, D., Xu, Y., Haizlip, K.M., . . . Janssen, P.M. (2010). Effects of dietary omega–3 fatty acids on ventricular function in dogs with healed myocardial infarctions: in vivo and in vitro studies. *American Journal of Physiology: Heart and Circulatory Physiology, 298*, H1219–H1228. doi: doi:10.1152/ajpheart.01065.2009

Bourre, J.M.E., Dumont, O.L., Piciotti, M.J., Clement, M.E., & Durand, G.A. (1997). Comparison of vegetable and fish oil in the provision of N-3 polyunsaturated fatty acids for nervous tissue and selected organs. *Journal of Nutritional Biochemistry, 8*(8), 472-478.

Brenna, J.T. (2002). Efficiency of conversion of [alpha]-linolenic acid to long chain n-3 fatty acids in man. *Current Opinion in Clinical Nutrition & Metabolic Care, 5*(2), 127-132.

Brenna, J.T., Salem Jr., N., Sinclair, A.J., & Cunnane, S.C. (2009). α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. [Review]. *Prostaglandins Leukotrienes and Essential Fatty Acids, 80*, 85-91.

Brenner, R.R. (1974). The oxidative desaturation of unsaturated fatty acids in animals. *Molecular and cellular biochemistry, 3*(1), 41-52.

Brouwer, I.A., Heeringa, J., Geleijnse, J.M., Zock, P.L., & Witteman, J.C.M. (2006a). Intake of very long-chain n-3 fatty acids from fish and incidence of atrial fibrillation. The Rotterdam Study. *American Heart Journal, 151*(4), 857-862.

Brouwer, I.A., Zock, P.L., Camm, A.J., Bocker, D., Hauer, R.N.W., Wever, E.F.D., . . . Schouten, E.G. (2006b). Effect of fish oil on ventricular tachyarrhythmia and death in patients with implantable cardioverter defibrillators. The study on omega-3 fatty acids and ventricular arrhythmia (SOFA) randomized trial. *Journal of the American Medical Association, 295*(22), 2613-2619.

Brouwer, I.A., Zock, P.L., van Amelsvoort, L.G.P.M., Katan, M.B., & Schouten, E.G. (2002). Association between n-3 fatty acid status in blood and electrocardiographic predictors of arrhythmia risk in healthy volunteers. *The American Journal of Cardiology, 89*, 629-631.

Brown, A.J., Pang, E., & Roberts, D.C.K. (1991). Erythrocyte eicosapentaenoic acid versus docosahexaenoic acid as a marker for fish and fish oil consumption. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 44*, 103-106.

Burdge, G.C., & Calder, P.C. (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reproductive Nutrition and Development, 45*(5), 581-597.

Burr, G., & Burr, M.M. (1929). A new deficiency disease produced by the rigid exclusion of fat from the diet. *The Journal of Biological Chemistry, 82*(2), 345-367.

Burr, G., & Burr, M.M. (1930). On the nature and role of the fatty acids essential in nutrition. *Journal of Biological Chemistry, 86*(2), 587-621.

Burr, M.L., Gilbert, J.F., Holliday, R.M., Elwood, P.C., Fehily, A.M., Rogers, S., . . . Deadman, N.M. (1989). Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and Reinfarction Trial (DART). *The Lancet, 334*(8666), 757-761.

Calder, P.C. (2004). n-3 fatty acids and cardiovascular disease: evidence explained and mechanisms explored. [Review]. *Clinical Science, 107*, 1-11.

Calò, L., Bianconi, L., Colivicchi, F., Lamberti, F., Loricchio, M.L., de Ruvo, E., . . . Santini, M. (2005). N-3 fatty acids for the prevention of atrial fibrillation after coronary artery bypass surgery: A randomized, controlled trial. *Journal of American College of Cardiology, 45*(10), 1723-1728.

Canton, M., Menazza, S., Sheeran, F.L., Polverino de Laureto, P., Di Lisa, F., & Pepe, S. (2011). Oxidation of Myofibrillar Proteins in Human Heart Failure. *Journal of the American College of Cardiology, 57*(3), 300-309. doi: 10.1016/j.jacc.2010.06.058

Cao, J., Schwichtenberg, K.A., Hanson, N.Q., & Tsai, M.Y. (2006). Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plamsa phospholipids. *Clinical Chemistry, 52*(12), 2265-2272.

Charnock, J.S. (1985). Dietary fats and cardiac function. *Proceedings of the Nutrition Society of Australia, 10*, 25-33.

Charnock, J.S., Abeywardena, M.Y., & McLennan, P.L. (1989). Tissue specific differences in the fatty acid composition of the marmoset monkey (*Callithrix jacchus*). *Comparative Biochemistry & Physiology A-Comparative Physiology, 92*(3), 299-304.

Charnock, J.S., Abeywardena, M.Y., Poletti, V.M., & McLennan, P.L. (1992). Differences in fatty acid composition of various tissues of the marmoset monkey (Callithrix jacchus) after different lipid supplemented diets. *Comparative Biochemistry and Physiology A- Comparative Physiology, 101*(2), 387-393.

Charnock, J.S., Abeywardena, M.Y., Tan, D., & McLennan, P.L. (1991). Omega-3 and Omega-6 PUFA's have different effects on the phospholipid fatty acid composition of rat myocardial muscle when added to a saturated fatty acid dietary supplement. *Nutrition Research, 11*, 1013-1024.

Charnock, J.S., Dryden, W.F., McMurchie, E.J., Abeywardena, M.Y., & Russell, G.R. (1983). Differences in the fatty acid composition of atrial and ventricular phospholipids of rat heart following standard and lipid-supplemented diets. *Comparative Biochemistry & Physiology - B: Comparative Biochemistry, 75*(1), 47-52.

Charnock, J.S., McIntosh, G.H., Abeywardena, M.Y., & Russell, G.R. (1985a). Changes in fatty acid composition of the cardiac phospholipids of the cotton-eared marmoset (Callithrix jacchus) after feeding different lipid supplements. *Annals of Nutrition & Metabolism, 29*(2), 83-94.

Charnock, J.S., McLennan, P.L., Abeywardena, M.Y., & Russell, G.R. (1985b). Altered levels of n-6/n-3 fatty acids in rat heart and storage fat following variable dietary intake of linoleic acid. *Annals of Nutrition & Metabolism, 29*(5), 279-288.

Childs, C.E., Romeu-Nadal, M., Burdge, G.C., & Calder, P.C. (2008). Gender differences in the n-3 fatty acid content of tissues. *Proceedings of the Nutrition Society, 67*(2008), 19-27. doi: 10.1017/S0029665108005983

Christensen, J.H. (2011). Omega-3 Polyunsaturated Fatty Acids and Heart Rate Variability. [Review]. *Frontiers in Physiology, 2*. doi: 10.3389/fphys.2011.00084

Christensen, J.H., Gustenhoff, P., Korup, E., Aaroe, J., Toft, E., Moller, J.M., . . . Schmidt, E.B. (1997). [n-3 polyunsaturated fatty acids, heart rate variability and ventricular arrhythmias in post-AMI-patients. A clinical controlled trial]. *Ugeskrift for Laeger, 159*(37), 5525-5529.

Christensen, J.H., Riahi, S., Schmidt, E.B., Molgaard, H., Pedersen, A.K., Heath, F., . . . Toft, E. (2005). n-3 Fatty acids and ventricular arrhythmias in patients with ischaemic heart disease and implantable cardioverter defibrillators. *Europace, 7*(4), 338-344. doi: 10.1016/j.eupc.2005.02.118

Clandinin, M.T. (1997). Influence of diet fat on membranes. In J.P. Inc. (Ed.), *Principles of Medical Biology, Volume 7A: Membranes and Cell Signaling, Chapter 5* (pp. 93-119).

Clandinin, M.T., Cheema, S., Field, C.J., Garg, M.L., Venkatraman, J., & Clandinin, T.R. (1991). Dietary fat: exogenous determination of membrane structure and cell function. *FASEB Journal, 5*, 2761-2769.

Clark, R.A., McLennan, S., Dawson, A., Wilkinson, D., & Stewart, S. (2004). Uncovering a hidden epidemic: a study of the current burden of heart failure in Australia. *Heart Lung and Circulation, 13*, 266-273. doi: 10.1016/j.hlc.2004.06.007

Connor, S.L., Zhu, N., Anderson, G.J., Hamill, D., Jaffe, E., Carlson, J., & Connor, W.E. (2000). Cheek cell phospholipids in human infants: a marker of docosahexaenoic and arachidonic acids in the diet, plasma, and red blood cells. *American Journal of Clinical Nutrition., 71*(1), 21-27.

Cordain, L., Eaton, S.B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B.A., . . . Brand-Miller, J. (2005). Origins and evolution of the western diet: health implications for the 21st century. *American Journal of Clinical Nutrition, 81*, 341-354.

Crystal, E., Connolly, S.J., Sleik, K., Ginger, T.J., & Yusuf, S. (2002). Interventions on prevention of postoperative atrial fibrillation in patients undergoing heart surgery: A meta-analysis. *Circulation, 106*, 75-80.

Dallongeville, J., Yarnell, J., Ducimetiere, P., Arveiler, D., Ferrieres, J., Montaye, M., . . . Amouyel, P. (2003). Fish consumption is associated with lower heart rates. *Circulation., 108*(7), 820-825.

Dawber, T.R., Meadors, G.F., & Moore, F.E. (1951). Epidemiological Approaches to Heart Disease: The Framingham Study. *Am J Public Health Nations Health, 41*(3), 279–286.

Dawber, T.R., Moore, F.E., & Mann, G.V. (1957). Measuring the Risk of Coronary Heart Disease in Adult Population Groups II. Coronary Heart Disease in the Framingham Study. *Am J Public Health Nations Health, 47*(4 (Pt 2)), 4–24.

Den Ruijter, H.M., Verkerk, A.O., Schumacher, C.A., Houten, S.M., Belterman, C.N.W., Baartscheer, A., . . . Coronel, R. (2012). A Diet Rich in Unsaturated Fatty Acids Prevents Progression Toward Heart Failure in a Rabbit Model of Pressure and Volume Overload: Clinical Perspective. *Circulation: Heart Failure, 5*(3), 376-384.

Du, X.-J., Fang, L., & Kiriazis, H. (2006). Sex dimorphism in cardiac pathophysiology: Experimental findings, hormonal mechanisms, and molecular mechanisms. *Pharmacology & Therapeutics, 111*(2006), 434-475. doi: 10.1016/j.pharmthera.2005.10.016

Duda, M.K., O'Shea, K.M., & Stanley, W.C. (2009). n-3 polyunsaturated fatty acid supplementation for the treatment of heart failure: mechanisms and clinical potential. *Cardiovascular Research, 84*, 33-41.

Dyerberg, J. (1986). Linolenate-derived Polyunsaturated Fatty Acids and Prevention of Atherosclerosis. *Nutrition Reviews, 44*(4), 125-134.

Dyerberg, J., & Bang, H.O. (1978a). Dietary fat and thrombosis. *Lancet, 311*, 152.

Dyerberg, J., & Bang, H.O. (1982). A hypothesis on the development of acute myocardial infarction in Greenlanders. *Scandinavian Journal of Clinical and Laboratory Investigation, 42*(Suppl 161), 7-13.

Dyerberg, J., Bang, H.O., & Hjorne, N. (1975). Fatty acid composition of the plasma lipids in Greenland Eskimos. *The American Journal of Clinical Nutrition, 28*(9), 958-966.

Dyerberg, J., Bang, H.O., & Hjorne, N. (1989). Coronary heart disease in Greenland Inuit: A paradox. Implications for Western diet patterns. *Arctic Medical Research, 48*(2), 47-54.

Dyerberg, J., Bang, H.O., Stoffersen, E., Moncada, S., & Vane, J.R. (1978b). Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? *Lancet, 312*(8081), 117-119.

Emilsson, A., & Gudbjarnason, S. (1981). Changes in fatty acyl chain composition of rat heart phospholipids induced by noradrenaline. *Biochimica et Biophysica Acta, 664*(1), 82-88.

Emilsson, A., & Gudbjarnason, S. (1983). Reversible alterations in fatty acid profile of glycerophospholipids in rat heart muscle induced by repeated norepinephrine administration. *Biochimica et Biophysica Acta, 750*(1), 1-6.

Farquharson, A.L., Metcalf, R.G., Sanders, P., Stuklis, R., Edwards, J.R.M., Gibson, R.A., . . . Young, G.D. (2011). Effect of Dietary Fish Oil on Atrial Fibrillation After Cardiac Surgery. *The American Journal of Cardiology, 108*(6), 851-856.

Finzi, A.A., Latini, R., Barlera, S., Rossi, M.G., Ruggeri, A., Mezzani, A., . . . Marchioli, R. (2011). Effects of n-3 polyunsaturated fatty acids on malignant ventricular arrhythmias in patients with chronic heart failure and implantable cardioverter-defibrillators: A substudy of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Insufficienza Cardiaca (GISSI-HF) trial. *American Heart Journal, 161*(2), 338-343.e331. doi: 10.1016/j.ahj.2010.10.032

Fletcher, R.F. (1972). Lipids of human myocardium. *Lipids, 7*(11), 728-732.

Folch, J., Lees, M., & Sloane Stanley, G.H. (1956). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biochemistry, 226*, 497-509.

Frost, L., & Vestergaard, P. (2005). n–3 Fatty acids consumed from fish and risk of atrial fibrillation or flutter: the Danish Diet, Cancer, and Health Study. *The American Journal of Clinical Nutrition, 81*(1), 50-54.

Garg, M.L., Leitch, J., Robert, J.B., & Garg, R. (2006). Long-chain n-3 polyunsaturated fatty acid incorporation into human atrium following fish oil supplemetation. *Lipids, 41*(12), 1127-1132.

Gebauer, S.K., Psota, T.L., Harris, W.S., & Kris-Etherton, P.M. (2006). n-3 fatty acid dietary recommendations and food sources to achieve essentiality and

cardiovascular benefits. *American Journal of Clinical Nutrition, 83*(6), 1526S-1535S.

Geelen, A., Brouwer, I.A., Schouten, E.G., Maan, A.C., Katan, M.B., & Zock, P.L. (2005). Effects of n-3 fatty acids from fish on premature ventricular complexes and heart rate in humans. *American Journal of Clinical Nutrition, 81*(2), 416-420.

Geelen, A., Brouwer, I.A., Zock, P.L., & Katan, M.B. (2004). Antiarrhythmic effects of n-3 fatty acids: evidence from human studies. *Current Opinion in Lipidology, 15*(1), 25-30.

Gloster, J., & Harris, P. (1969). The lipid composition of mitochondrial and microsomal fractions of human myocardial homogenates. *Cardiovascular Research, 3*, 45-51.

Gloster, J., & Harris, P. (1970). The lipid composition of mitochondrial and microsomal fractions from human ventricular myocardium. *Journal of Molecular and Cellular Cardiology, 1*, 459-465.

Griffin, B.A. (2008). How relevant is the ratio of dietary n-6 to n-3 polyunsaturated fatty acids to cardiovascular disease risk? Evidence from the OPTILIP study. *Current Opinion in Lipidology, 19*(1), 57-62.

Grimsgaard, S., Bonaa, K.H., Hansen, J.B., & Myhre, E.S. (1998). Effects of highly purified eicosapentaenoic acid and docosahexaenoic acid on hemodynamics in humans. *American Journal of Clinical Nutrition, 68*, 52-59.

Gudbjarnason, S. (1975). Prostaglandins and polyunsaturated fatty acids in heart muscle. *Journal of Molecular and Cellular Cardiology, 7*(7), 443-449.

Gudbjarnason, S. (1980). Pathophysiology of long-chain polyene fatty acids in heart muscle. *Nutrition and Metabolism, 24*(Suppl 1), 142-146.

Gudbjarnason, S. (1989). Dynamics of n-3 and n-6 fatty acids in phospholipids of heart muscle. *Journal of Internal Medicine, 225*(Suppl 731), 117-128.

Gudbjarnason, S., & Benediktsdottir, V.E. (1995). Coregulation of adrenoceptors and the lipid environment in heart muscle during repeated adrenergic stimulation. *Journal of Molecular & Cellular Cardiology, 27*(1), 243-251.

Gudbjarnason, S., & Benediktsdottir, V.E. (1996). Regulation of beta-adrenoceptor properties and the lipid milieu in heart muscle membranes during stress. *Molecular & Cellular Biochemistry, 164*, 137-143.

Gudbjarnason, S., Doell, B., & Oskardottir, G. (1978a). Modification of cardiac phospholipids and catecholamine stress tolerance. In C. de Duve & O. Hayaishi (Eds.), *International Symposium on Tocopherol, Oxygen, and Biomembranes. Lake Yamanaka, Japan.* (Vol. Tocopherol, Oxygen, and Biomembranes, pp. 297-310). Amsterdam: Elsevier Science.

Gudbjarnason, S., Doell, B., & Oskarsdottir, G. (1978b). Docosahexaenoic acid in cardiac metabolism and function. *Acta Biologica et Medica Germanica., 37*(5-6), 777-784.

Gudbjarnason, S., & Hallgrimsson, J. (1975a). The role of myocardial membrane lipids in the development of cardiac necrosis. *Acta Medica Scandinavia, S587*, 17-26.

Gudbjarnason, S., & Hallgrimsson, J. (1976). Prostaglandins and polyunsaturated fatty acids in heart muscle. *Acta biologica et medica Germanica, 35*, 1069-1080.

Gudbjarnason, S., & Oskarsdottir, G. (1975b). Changes in fatty acid composition of cardiac lipids accompanying myocardial necrosis. *Recent Advances in Studies on Cardiac Structure & Metabolism, 6*, 193-203.

Gudbjarnason, S., & Oskarsdottir, G. (1977). Modification of fatty acid composition of rat heart lipids by feeding cod liver oil. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 487*(1), 10-15.

Gudbjarnason, S., Oskarsdottir, G., Doell, B., & Hallgrimsson, J. (1978c). Myocardial membrane lipids in relation to cardiovascular disease. *Advances in Cardiology, 25*, 130-144.

Haissaguerre, M., Jais, P., Shah, D., Takahashi, A., Hocini, M., Quiniou, G., . . . Clementy, J. (1998). Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *The New England Journal of Medicine, 339*(10), 659-666.

Harris, W.S. (2007). Omega-3 fatty acids and cardiovascular disease: A case for omega-3 index as a new risk factor. *Pharmacological Research, 55*(3), 217-223.

Harris, W.S. (2009). The Omega-3 Index: From biomarker to risk marker to risk factor. *Current Atherosclerosis Reports, 11*(6), 411-417. doi: 10.1007/s11883-009-0062-2

Harris, W.S. (2010). Omega-6 and omega-3 fatty acids: Partners in prevention. *Current Opinion in Clinical Nutrition and Metabolic Care, 13*(2), 125-129. doi: 10.1097/MCO.0b013e3283357242

Harris, W.S., Kris-Etherton, P.M., & Harris, B.A. (2008a). Intakes of long-chain omega-3 fatty acid associated with reduced risk for death from coronary heart disease in healthy adults. *Current Atherosclerosis Reports, 10*(6), 503-506. doi: 10.1007/s11883-008-0078-z

Harris, W.S., Miller, M., Tighe, A.P., Davidson, M.H., & Schaefer, E.J. (2008b). Omega-3 fatty acids and coronary heart disease risk: Clinical and mechanistic perspectives. *Atherosclerosis, 197*(2008), 12-24.

Harris, W.S., Pottala, J.V., Vasan, R.S., Larson, M.G., & Robins, S.J. (2012). Changes in Erythrocyte Membrane Trans and Marine Fatty Acids between 1999 and 2006 in Older Americans. *Journal of Nutrition, 142*(7), 1297-1303. doi: 10.3945/jn.112.158295

Harris, W.S., Sands, S.A., Windsor, S.L., Ali, H.A., Stevens, T.L., Magalski, A., . . . Borkon, A.M. (2004a). Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: Correlation with erythrocytes and response to supplementation. *Circulation, 110*(12), 1645-1649.

Harris, W.S., & von Schacky, C. (2004b). The Omega-3 Index: A new risk factor for death from coronary heart disease? [original]. *Preventive Medicine, 39*, 212-220. doi: 10.1016/j.ypmed.2004.02.030

Hasenfuss, G. (1998). Animal models of human cardiovascular disease, heart failure and hypertrophy. *Cardiovascular Research, 39*(1), 60-76.

Heckers, H., Korner, M., Tuschen, T.W.L., & Melcher, F.W. (1977). Occurrence of individual trans-isomeric fatty acids in human myocardium, jejunum and aorta in relation to different degrees of atherosclerosis. *Atherosclerosis, 28*, 389-398.

Heidarsdottir, R., Arnar, D.O., Skuladottir, G.V., Torfason, B., Edvardsson, V., Gottskalksson, G., . . . Indridason, O.S. (2010). Does treatment with n-3 polyunsaturated fatty acids prevent atrial fibrillation after open heart surgery? *Europace, 12*(3), 356-363. doi: 10.1093/europace/eup429

Helge, J.W., Ayre, K.J., Hulbert, A.J., Kiens, B., & Storlien, L.H. (1999). Regular exercise modulates muscle membrane phospholipid profile in rats. *Journal of Nutrition, 129*(9), 1636-1642.

Helge, J.W., Wu, B.J., Willer, M., Daugaard, J.R., Storlien, L.H., & Kiens, B. (2001). Training affects muscle phospholipid fatty acid composition in humans. *Journal of Applied Physiology, 90*(2), 670-677.

Hock, C.E., Holahan, M.A., & Reibel, D.K. (1987). Effect of dietary fish oil on myocardial phospholipids and myocardial ischemic damage. *American Journal of Physiology: Heart and Circulation Physiology, 252*(21), H554-H560.

Holman, R.T. (1986). Control of polyunsaturated acids in tissue lipids. *Journal of the American College of Nutrition, 5*(2), 183-211.

Holman, R.T., & Johnson, S. (1981). Changes in essential fatty acid profile of serum phospholipids in human disease. *Annual Reviews*, 67-73.

Hooper, L., Thompson, R.L., Harrison, R.A., Summerbell, C.D., Ness, A.R., Moore, H.J., . . . Davey Smith, G. (2006). Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *British Medical Journal, 332*(7544), 752 (751-759). doi: 10.1136/bmj.38755.366331.2F

Horrobin, D.F. (1993). Fatty acid metabolism in health and disease: the role of delta-6-desaturase. *The American Journal of Clinical Nutrition, 57*(5), 732S-736S.

Howe, P., Buckley, J., & Meyer, B. (2007). Long-chain omega-3 fatty acids in red meat. *Nutrition & Dietetics, 64*(Supplement 4), S135-S139.

Hu, F.B., Manson, J.E., & Willett, W.C. (2001). Types of dietary fat and risk of coronary heart disease: A critical review. *Journal of the American College of Nutrition, 20*(1), 5-19.

Huggins, C.E., Curl, C.L., Patel, R., McLennan, P.L., Theiss, M.L., Pedrazzini, T., . . . Delbridge, L.M. (2009). Dietary fish oil is antihypertrophic but does not enhance postischemic myocardial function in female mice. *American Journal of Physiology, Heart and Circulation Physiology, 296*, H957-H966.

Jahangiri, A., Leifert, W.R., Patten, G.S., & McMurchie, E.J. (2000). Termination of asynchronous contractile activity in rat atrial myocytes by n-3 polyunsaturated fatty acids. *Molecular and cellular biochemistry, 206*, 33-41.

Jordan, H., Matthan, N., Chung, M., Balk, E.M., Chew, P., Kupelnick, B., . . . Lau, J. (2004). Effects of Omega-3 Fatty Acids on Arrhythmogenic Mechanisms in Animal and Isolated Organ/Cell Culture Studies. *AHRQ Publication No 04-E011-2. Evidence Report/Technology Assessment No. 92. Prepared by Tufts-New England Medical Center Evidence-based Practice Center., Number 92*, 1-228.

Jump, D.B. (2002). The biochemistry of n-3 polyunsaturated fatty acids. *The Journal of Biological Chemistry, 277*(11), 8755-8758. doi: 10.1074/jbc.R100062200

Kajstura, J., Gurusamy, N., Ogo´rek, B., Goichberg, P., Clavo-Rondon, C., Hosoda, T., . . . Anversa, P. (2010). Myocyte Turnover in the Aging Human Heart. *Circulation Research, 107*, 1374-1386.

Keys, A. (1956). The diet and the development of coronary heart disease. *Journal of Chronic Disease, 4*(4), 364-380.

Keys, A., & Grande, F. (1957). Role of Dietary Fat in Human Nutrition: Ill-Diet and the Epidemiology of Coronary Heart Disease. *American Journal of Public Health, 47*, 1521-1530.

Kowey, P.R., Reiffel, J.A., Ellenbogen, K.A., Naccarelli, G.V., & Pratt, C.M. (2010). Efficacy and Safety of Prescription Omega-3 Fatty Acids for the Prevention of Recurrent Symptomatic Atrial Fibrillation. *The Journal of the American Medical Association, 304*(21), 2363-2372. doi: 10.1001/jama.2010.1735

Kris-Etherton, P.M., Harris, W.S., Appel, L.J., & for the Nutrition, C. (2002). Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. *Circulation, 106*(21), 2747-2757. doi: 10.1161/01.cir.0000038493.65177.94

Kris-Etherton, P.M., Taylor, D.S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., . . . Etherton, T.D. (2000). Polyunsaturated fatty acids in the food chain in the United States. *American Journal of Clinical Nutrition, 71*(Suppl), 179S-188S.

Kromhout, D., Bosschieter, E.B., & Coulander, C.L. (1985). The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *The New England Journal of Medicine, 312*(19), 1205-1209.

Krum, H., Jelinek, M., Stewart, S., Sindone, A., Atherton, J.J., & Hawkes, A. (2006). Guidelines for the prevention, detection and management of chronic heart failure in Australia, 2006. *National Heart Foundation of Australia and the Cardiac Society of Australia and New Zealand*, 1-80.

Kumar, S., Sutherland, F., Teh, A.W., Heck, P.M., Lee, G., Garg, M.L., & Sparks, P.B. (2011). Effects of chronic omega-3 polyunsaturated fatty acid supplementation on human pulmonary vein and left atrial electrophysiology in paroxysmal atrial fibrillation. *AMerican Journal of Cardiology, 108*, 531-535. doi: 10.1016/j.amjcard.2011.03.082

Lavie, C.J., Milani, R.V., Mehra, M.R., & Ventura, H.O. (2009). Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Diseases. *Journal of the American College of Cardiology, 54*(7), 585-594.

Leaf, A., Kang, J.X., Xiao, Y.-F., & Billman, G.E. (2003). Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation, 107*, 2646-2652.

Lee, H.J., O'Keefe, J.H., Lavie, C.J., Marchioli, R., & Harris, W.S. (2008). Omega-3 fatty acids for cardioprotection. *Mayo Clinic Proceedings, 83*(3), 324-332.

Leong, J.Y., van der Merwe, J., Pepe, S., Bailey, M., Perkins, A., Lymbury, R., . . . Rosenfeldt, F. (2010). Perioperative metabolic therapy improves redox status and outcomes in cardiac surgery patients: A randomised trial. [Article]. *Heart Lung and Circulation, 19*(10), 584-591. doi: 10.1016/j.hlc.2010.06.659

London, B., Albert, C.M., Anderson, M.E., Giles, W.R., Van Wagoner, D.R., Balk, E., . . . Lathrop, D.A. (2007). Omega-3 Fatty Acids and Cardiac Arrhythmias: Prior Studies and Recommendations for Future Research. *Circulation: A Report from the National Heart, Lung, and Blood Institute and Office of Dietary Supplements Omega-3 Fatty Acids and Their Role in Cardiac Arrhythmogenesis Workshop, 116*(10), e320-335. doi: 10.1161/circulationaha.107.712984

Lund, E.K., Harvey, L.J., Ladha, S., Clark, D.C., & Johnson, I.T. (1999). Effects of dietary fish oil supplementation on the phospholipid composition and fluidity of cell membranes from human volunteers. *Annals of Nutrition and Metabolism, 43*(5), 290-300.

Luxon, B.A., Holly, D.C., Milliano, M.T., & Weisiger, R.A. (1998). Sex differences in multiple steps in hepatic transport of palmitate support a balanced uptake mechanism. *American Journal of Physiology (Gastrointestinal and Liver Physiology), 274*(37), G52-G61.

Marchioli, R. (2002). Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction. Time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation, 105*, 1897-1903.

Marchioli, R., Barzi, F., Bomba, E., Chieffo, C., Di Gregorio, D., Di Mascio, R., . . . Investigators, G.-P. (2002). Early protection against sudden death by n-3

polyunsaturated fatty acids after myocardial infarction: Time-course analysis of the results of the GISSI-Prevenzione. *Circulation, 105*(16), 1897-1903.

Marchioli, R., Bomba, E., Chieffo, C., Maggioni, A.P., Schweiger, C., & Tognoni, G. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *The Lancet, 354*(9177), 447-455.

McLennan, P.L. (2001). Myocardial membrane fatty acids and the antiarrhythmic actions of dietary fish oil in animal models. *Lipids, 36*(Suppl 1), S111-S114.

McLennan, P.L. (2004). Omega-3 polyunsaturated fatty acid prevention of cardiac arrhythmia and sudden death: cellular or circulating? *Current Topics in Nutriceutical Research, 2*(1), 101-111.

McLennan, P.L., & Abeywardena, M.Y. (2005). Membrane basis for fish oil effects on the heart: Linking natural hibernators to prevention of human sudden cardiac death. *Journal of Membrane Biology, 206*, 85-102.

McLennan, P.L., Abeywardena, M.Y., & Charnock, J.S. (1985). Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Canadian Journal of Physiology and Pharmacology, 63*, 1411-1417.

McLennan, P.L., Abeywardena, M.Y., & Charnock, J.S. (1988). Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion. *American Heart Journal, 116*(3), 709-717.

McLennan, P.L., Abeywardena, M.Y., & Charnock, J.S. (1989). The influence of age and dietary fat in an animal model of sudden cardiac death. *Australian and New Zealand Journal of Medicine, 19*, 1-5.

McLennan, P.L., Abeywardena, M.Y., & Charnock, J.S. (1990). Reversal of the arrhythmogenic effects of long-term saturated fatty acid intake by dietary n-3 and n-6 polyunsaturated fatty acids. *American Journal of Clinical Nutrition, 51*, 53-58.

McLennan, P.L., Abeywardena, M.Y., Dallimore, J.A., & Raederstorff, D. (2012). Dietary fish oil preserves cardiac function in the hypertrophied rat heart. *British Journal of Nutrition, 108*(4), 645-654. doi: 10.1017/S0007114511005915

McLennan, P.L., Bridle, T.M., Abeywardena, M.Y., & Charnock, J.S. (1993). Comparative efficacy of n-3 and n-6 polyunsaturated fatty acids in modulating ventricular fibrillation threshold in marmoset monkeys. *American Journal of Clinical Nutrition, 58*(5), 666-669.

McLennan, P.L., Howe, P., Abeywardena, M.Y., Muggli, R., Raederstorff, D., Mano, M., . . . Head, R. (1996). The cardiovascular protective role of docosahexaenoic acid. *European Journal of Pharmacology, 300*, 83-89.

McLennan, P.L., Owen, A.J., Slee, E.L., & Theiss, M.L. (2007). Myocardial function, ischaemia and n-3 polyunsaturated fatty acids: A membrane basis. *Journal of Cardiovascular Medicine, 8*(Suppl 1), 15-18.

Metcalf, R.G., Cleland, L.G., Gibson, R.A., Roberts-Thomson, K.C., Edwards, J.R.M., Sanders, P., . . . Young, G.D. (2010). Relation between blood and atrial fatty acids in patients undergoing cardiac bypass surgery. *American Journal of Clinical Nutrition, 91*(3), 528-534.

Metcalf, R.G., James, M.J., Gibson, R.A., Edwards, J.R.M., Stubberfield, J., Stuklis, R., . . . Cleland, L.G. (2007). Effects of fish-oil supplementation on myocardial fatty acids in humans. *American Journal of Clinical Nutrition, 85*(5), 1222-1228.

Meyer, B.J., Mann, N.J., Lewis, J.L., Milligan, G.C., Sinclair, A.J., & Howe, P.R.C. (2003). Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids, 38*(4), 391-398.

Milte, C.M., Coates, A.M., Buckley, J.D., Hill, A.M., & Howe, P.R.C. (2008). Dose-dependent effects of docosahexaenoic acid-rich fish oil on erythrocyte docosahexaenoic acid and blood lipid levels. *British Journal of Nutrition, 99*, 1083-1088.

Mori, T.A., Bao, D.Q., Burke, V., Puddey, I.B., & Beilin, L.J. (1999). Docosahexaenoic acid but not eicosapentaenoic acid lowers ambulatory blood pressure and heart rate in humans. *Hypertension, 34*(2), 253-260.

Morris, M.C., Manson, J.E., Rosner, B., Buring, J.E., Willett, W.C., & Hennekens, C.H. (1995). Fish Consumption and Cardiovascular Disease in the Physicians' Health Study: A Prospective Study. *American Journal of Epidemiology, 142*(2), 166-175.

Morris, M.C., Sacks, F., & Rosner, B. (1993). Does fish oil lower blood pressure? A meta-analysis of controlled trials. *Circulation, 88*(2), 523-533.

Mozaffarian, D. (2007). Fish, n-3 fatty acids, and cardiovascular haemodynamics. *Journal of Cardiovascular Medicine, 8*, S23-S26.

Mozaffarian, D., Bryson, C.L., Lemaitre, R.N., Burke, G.L., & Siscovick, D.S. (2005a). Fish intake and risk of incident heart failure. *Journal of the American College of Cardiology, 45*(12), 2015-2021.

Mozaffarian, D., Geelen, A., Brouwer, I.A., Geleijnse, J.M., Zock, P.L., & Katan, M.B. (2005b). Effect of fish oil on heart rate in humans: A meta-analysis of randomized controlled trials. *Circulation, 112*(13), 1945-1952.

Mozaffarian, D., Gottdiener, J.S., & Siscovick, D.S. (2006a). Intake of Tuna or Other Broiled or Baked Fish Versus Fried Fish and Cardiac Structure, Function, and Hemodynamics. *AMerican Journal of Cardiology, 97*, 216 -222.

Mozaffarian, D., Prineas, R.J., Stein, P.K., & Siscovick, D.S. (2006b). Dietary fish and n-3 fatty acid intake and cardiac electrocardiographic parameters in humans. *Journal of the American College of Cardiology, 48*(3), 478-484.

Mozaffarian, D., Psaty, B.M., Rimm, E.B., Lemaitre, R.N., Burke, G.L., Lyles, M.F., . . . Siscovick, D.S. (2004). Fish intake and risk of incident atrial fibrillation. *Circulation, 110*(4), 368-373.

Mozaffarian, D., Stein, P.K., Prineas, R.J., & Siscovick, D.S. (2008). Dietary fish and omega-3 fatty acid consumption and heart rate variability in US adults. *Circulation, 117*(9), 1130-1137.

Murnaghan, M.F. (1981). Effect of fatty acids on the ventricular arrhythmia threshold in the isolated heart of the rabbit. *British Journal of Pharmacology, 73*(4), 909-915.

Murphy, G.J., Reeves, B.C., Rogers, C.A., Rizvi, S.I.A., Culliford, L., & Angelini, G.D. (2007). Increased mortality, postoperative morbidity, and cost after red blood cell transfusion in patients having cardiac surgery. *Circulation, 116*(22), 2544-2552. doi: 10.1161/circulationaha.107.698977

Murry, C.E., Jennings, R.B., & Reimer, K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation, 74*(5), 1124-1136.

Nair, S.S.D., Leitch, J., Falconer, J., & Garg, M.L. (1999). Cardiac (n-3) non-esterified fatty acids are selectively increased in fish oil-fed pigs following myocardial ischaemia. *The Journal of Nutrition, 129*(8), 1518-1523.

Nakamura, Y., Ueshima, H., Okamura, T., Kadowaki, T., Hayakawa, T., Kita, Y., . . . Okayama, A. (2005). Association between fish consumption and all-cause and cause-specific mortality in Japan: NIPPON DATA80, 1980-99. *The American Journal of Medicine, 118*(3), 239-245.

Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., & Sprecher, H. (1979). Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proceedings of the National Academy of Sciences, 76*(2), 944-948.

Nelson, A.M. (1972). Diet therapy in coronary disease: Effect on mortality of high protein, high seafood, fat-controlled diet. *Geriatrics, 12*, 103-116.

NHFA. (2006). *Guidelines for the prevention, detection and management of chronic heart failure in Australia, 2006.*: National Heart Foundation of Australia and the Cardiac Society of Australia and New Zealand.

NHFA. (2008a). *Atrial Fibrillation*. National Heart Foundation of Australia, Heart Health Information Service.

NHFA. (2008b). Getting your omega-3s each week? *National Heart Foundation of Australia, CON-048*, 1-4.

NHFA. (2008c). *Heart Foundation Position Statement: Fish, fish oils, n-3 polyunsaturated fatty acids and cardiovascular health*. National Heart Foundation of Australia.

NHMRC. (2003). Dietary guidelines for Australian adults. *National Health and Medical Research Council*.

O'Keefe, J., J.H., Abuissa, H., Sastre, A., Steinhaus, D.M., & Harris, W.S. (2006). Effects of omega-3 fatty acids on resting heart rate, heart rate recovery after exercise, and heart rate variability in men with healed myocardial infarctions and depressed ejection fractions. [original]. *AMerican Journal of Cardiology, 97*, 1127-1130. doi: doi:10.1016/j.amjcard.2005.11.025

Ohlrogge, J.B., Emken, E.A., & Gulley, R.M. (1981). Human tissue lipids: occurence of fatty acid isomers from dietary hydrogenated oils. *Journal of Lipid Research, 22*, 955-960.

Ommen, S.R., Odell, J.A., & Stanton, M.S. (1997). Atrial arrhythmias after cardiothoracic surgery. *The New England Journal of Medicine, 336*(20), 1429-1434.

Owen, A.J., Peter-Przyborowska, B.A., Hoy, A.J., & McLennan, P.L. (2004). Dietary fish oil dose- and time-response effects on cardiac phospholipid fatty acid composition. *Lipids, 39*(10), 955-961.

Pepe, S., & McLennan, P.L. (1996). Dietary fish oil confers direct antiarrhythmic properties on the myocardium of rats. *Journal of Nutrition, 126*(1), 34-42.

Pepe, S., & McLennan, P.L. (2002). Cardiac Membrane Fatty Acid Composition Modulates Myocardial Oxygen Consumption and Post-Ischemic Recovery of Contractile Function. *Circulation, 105*(19), 2303-2308.

Pepe, S., & McLennan, P.L. (2007). (n-3) Long Chain PUFA Dose-Dependently Increase Oxygen Utilization Efficiency and Inhibit Arrhythmias after Saturated Fat Feeding in Rats. *Journal of Nutrition, 137*(11), 2377-2383.

Ray, S., Sengupta, K.P., & Chatterjee, G.C. (1979). Lipid composition of human myocardium. *Indian Journal of Experimental Biology, 17*, 988-989.

Reibel, D.K., O'Rourke, B., Foster, K.A., Hutchinson, H., Uboh, C.E., & Kent, R.L. (1986). Altered phospholipid-metabolism in pressure-overload hypertrophied hearts. *American Journal of Physiology: Heart, Circulation and Physiology, 250*(1), H1-H6.

Reiffel, J.A., & McDonald, A. (2006). Antiarrhythmic effects of omega-3 fatty acids. *AMerican Journal of Cardiology, 98*(4A), 50i-60i.

Rennison, J.H., & Van Wagoner, D.R. (2009). Impact of dietary fatty acids on cardiac arrhythmogenesis. *Circulation: Arrhythmia and Electrophysiology, 2*(4), 460-469.

Reynolds, M.R., Thosani, A.J., Pinto, D.S., & Josephson, M.E. (2010). Chapter 49. Sudden Cardiac Death. In V. Fuster, R.A. Walsh & R.A. Harrington (Eds.), *Hurst's The Heart, 13th edition*. US: McGraw-Hill.

Rocquelin, G., Guenot, L., Astorg, P.O., & David, M. (1989). Phospholipid content and fatty acid composition of human heart. *Lipids., 24*(9), 775-780.

Rocquelin, G., Guenot, L., Justrabo, E., Grynberg, A., & David, M. (1985). Fatty acid composition of human heart phospholipids: Data from 53 biopsy specimens. *Journal of Molecular and Cellular Cardiology, 17*, 769-773.

SACN. (2008). The Nutritional Wellbeing of the British Population. *Scientific Advisory Committee on Nutrition. Food Standards Agency and the Department of Health*.

Saito, M., Ueno, M., Kubo, K., & Yamaguchi, M. (1998). Dose-Response Effect of Dietary Docosahexaenoic Acid on Fatty Acid Profiles of Serum and Tissue Lipids in Rats. [Original]. *J. Agric. Food Chem, 46*(1), 184-193.

Saravanan, P., Bridgewater, B., West, A.L., O'Neill, S.C., Calder, P.C., & Davidson, N.C. (2009). Omega-3 Fatty Acid Supplementation Does Not Reduce Risk of Atrial Fibrillation After Coronary Artery Bypass Surgery. *Circulation: Arrhythmia and Electrophysiology, 3*(1), 46-53. doi: 10.1161/circep.109.899633

Sen, A., & Sengupta, K.P. (1981). Lipid profile of human hearts from Bengalee population. *Indian Journal of Medical Research, 74*, 739-745.

Serhan, C.N., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G., & Moussignac, R.-L. (2002). Resolvins: A family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *The Journal of Experimental Medicine, 196*(8), 1025-1037. doi: 10.1084/jem.20020760

Services, U.S.D.o.H.H. (2002). GRAS Notice Inventory GRN 109. Available from U.S. Food and Drug Administration GRAS Notice Inventory. (GRN No. 109). from U.S. Department of Health & Human Services http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=109

Sexton, P.T., Sinclair, A.J., O'Dea, K., Sanigorski, A.J., & Walsh, J. (1995). The relationship between linoleic acid level in serum, adipose tissue and myocardium in humans. *Asia Pacific Journal of Clinical Nutrition, 4*, 314-318.

Shekelle, R.B., Paul, O., Shryock, A.M., & Stamler, J. (1985). Fish consumption and mortality from coronary heart disease. *New England Journal of Medicine*, 820.

Shenolikar, I. (1980). Fatty acid profile of myocardial lipid in populations consuming different dietary fats. *Lipids, 15*(11), 980-982.

Siddiqui, R.A., Harvey, K.A., & Zaloga, G.P. (2008). Modulation of enzymatic activities by n-3 polyunsaturated fatty acids to support cardiovascular health. *Journal of Nutritional Biochemistry, 19*(7), 417-437.

Simon, G., & Rouser, G. (1969). Species variations in phospholipid class distribution of organs: Heart and skeletal muscle. *Lipids, 4*(6), 607-614.

Simopoulos, A.P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition, 54*, 438-463.

Siscovick, D.S., Lemaitre, R.N., & Mozaffarian, D. (2003). The fish story: a diet-heart hypothesis with clinical implications: n-3 polyunsaturated fatty acids, myocardial vulnerability, and sudden death. *Circulation, 107*, 2632-2634.

Siscovick, D.S., Raghunathan, T.E., King, I., Weinmann, S., Bovbjerg, V.E., Kushi, L.H., . . . Knopp, R.H. (2000). Dietary intake of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *The American Journal of Clinical Nutrition, 71(suppl)*, 208S-212S.

Siscovick, D.S., Raghunathan, T.E., King, I., Weinmann, S., Wicklund, K.G., Albright, J., . . . Knopp, R.H. (1995). Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *Journal of the American Medical Association, 274*(17), 1363-1367.

Slater-Jefferies, J.L., Hoile, S.P., Lillycrop, K.A., Townsend, P.A., Hanson, M.A., & Burdge, G.C. (2010). Effect of sex and dietary fat intake on the fatty acid composition of phospholipids and triacylglycerol in rat heart *Prostaglandins, Leukotrienes and Essential Fatty Acids, In Press, Corrected Proof, Available online 16 August 2010*. doi: doi:10.1016/j.plefa.2010.07.006

Slee, E.L., McLennan, P.L., Owen, A.J., & Theiss, M.L. (2010). Low dietary fish oil threshold for myocardial membrane n-3 PUFA enrichment independent of n-6 PUFA intake in rats. *Journal of Lipid Research, 51*, 1841-1848. doi: 10.1194/jlr.M004069

Spector, A.A. (1999). Essentiality of fatty acids. *Lipids, 34*(Supplement 1999), S1-S3.

Steinberg, D. (2004). An interpretive history of the cholesterol controversy: part I. *Journal of Lipid Research, 45*(9), 1583-1593. doi: 10.1194/jlr.R400003.JLR200

Stillwell, W., & Wassall, S.R. (2003). Docosahexaenoic acid: Membrane properties of a unique fatty acid. *Chemistry and Physics of Lipids, 126*(2003), 1-27. doi: 10.1016/S0009-3084(03)00101-4

Streppel, M.T., Ocke, M.C., Boshuizen, H.C., Kok, F.J., & Kromhout, D. (2008). Long-term fish consumption and n-3 fatty acid intake in relation to (sudden) coronary heart disease death: the Zutphen study. *European Heart Journal, 29*, 2024-2030. doi: 10.1093/eurheartj/ehn294

Strom, A. (1948). Examination into the diet of Norwegian families during the war-years, 1942-45. *Acta Medica Scandinavia Supplement, 164*, 1-47.

Strom, A., & Jensen, R.A. (1951). Mortality from circulatory diseases in Norway, 1940-1945. *The Lancet, 257*(6647), 126-129.

Stubbs, C.D., & Smith, A.D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica et Biophysica Acta, 779*(BBA 85255), 89-137.

Taubes, G. (2001). The soft science of dietary fat. *Science, 291*, 2536-2545.

Tavazzi, L., Maggioni, A.P., Marchioli, R., Barlera, S., Franzosi, M.G., Latini, R., . . . Tognoni, G. (2008a). Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): A randomised, double-blind, placebo-controlled trial. *Lancet, 372*(9645), 1223-1230.

Tavazzi, L., Tognoni, G., Maggioni, A.P., Marchioli, R., Latini, R., Franzosi, M.G., . . . Porcu, M. (2008b). Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): A randomised, double-blind, placebo-controlled trial. *The Lancet, 372*(9645), 1223-1230.

ten Hoor, F., & de Deckere, E.A.M. (1981). Essential fatty acids, prostaglandins and heart function. *Progress in Lipid Research, 20*(0), 423. doi: 10.1016/0163-7827(81)90074-6

U.S. Department of Agriculture, A.R.S. (2009). USDA National Nutrient Database for Standard Reference, Release 22. Available from U.S. Department of Agriculture, Agricultural Research Service. Nutrient Data Laboratory Home Page Retrieved 2009 Nov 16 http://www.ars.usda.gov/ba/bhnrc/ndl

USFDA, Department of Agriculture, U.S., & Department of Health and Human Services, U.S. (2010). *Dietary Guidelines for Americans, 2010.7th Edition*. Washington, DC.: U.S. Government Printing Office, December 2010.

Valagussa, F., Franzosi, M.G., Geraci, E., Mininni, N., Nicolosi, G.L., Santini, M., . . . Investigators, G.-P. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet, 354*(9177), 447-455.

van der Vusse, G.J., Roemen, T.H., Prinzen, F.W., Coumans, W.A., & Reneman, R.S. (1982). Uptake and tissue content of fatty acids in dog myocardium under normoxic and ischemic conditions. *Circulation Research, 50*(4), 538-546.

Von Schacky, C. (2009). Cardiovascular disease prevention and treatment. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 81*(2-3), 193-198. doi: 10.1016/j.plefa.2009.05.009

Wang, C., Harris, W.S., Chung, M., Lichtenstein, A.H., Balk, E.M., Kupelnick, B., . . . Lau, J. (2006). n-3 Fatty acids from fish or fish-oil supplements, but not (alpha)-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *American Journal of Clinical Nutrition, 84*(1), 5-17.

WHO. (2003). *Cardiovascular Diseases (CVD): Global strategy on diet, physical activity and health*.

WHO/FAO. (2004). The Joint WHO/FAO Expert Consultation on diet, nutrition and the prevention of chronic diseases: process, product and policy implications. *Public Health Nutrition, 7*(1A), 245-250.

Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishikawa, Y., . . . Investigators, J. (2007). Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised openlabel, blinded endpoint analysis. [Article]. *Lancet, 369*(9567), 1090-1098.

Appendix

APPENDIX A. Levels of n-3 fatty acids in Australian seafood: Fatty acid composition of seafood samples.

Fatty acid	Scientific Name	% Fat ± SD	Total Saturated	Total Mono-unsaturated	18:2n–6 †LA	20:4n–6 ‡AA	Total n–6	18:3n–3 §ALA	20:5n–3 §§EPA	22:5n–3 ¶DPA	22:6n–3 ††DHA	Total n–3
Atlantic Salmon	Salmo Salar	11.2 ± 0.28	26.3±0.54	34.3±0.18	0.2±0.00	1.2±0.21	7.3±0.02	1.5±0.01	8.6±0.15	5.2±0.05	17.1±0.47	31.8±0.73
Barramundi	Lates Calcarifer	1.2 ± 0.19	30.7±0.09	22.3±0.56	0.3±0.01	13.2±0.34	20.2±0.27	1.0±0.03	7.2±0.17	4.1±0.09	9.5±0.18	23.6±0.32
Boar	Pentaceropsis recurvirostris	0.9 ± 0.05	34.2±0.21	10.2±0.23	0.1±0.00	14.2±0.32	18.1±0.27	0.1±0.01	4.6±0.09	2.4±0.05	29.5±0.27	36.7±0.25
Blue Grenadier	Macruronus novaezelandiae	0.7 ± 0.01	30.0±0.03	27.9±2.46	0.2±0.02	2.1±0.18	3.8±0.02	0.3±0.04	4.5±0.08	2.4±0.05	31.4±2.47	37.9±2.47
European Carp	Cyprinus carpio	2.7 ± 0.08	42.4±0.39	17.7±0.56	0.1±0.02	5.8±0.37	8.2±0.40	0.1±0.03	4.7±0.13	2.1±0.08	23.8±0.60	30.8±0.39
Coral Trout	Plectropomus leopardus	0.7 ± 0.02	32.5±0.30	12.0±0.83	0.2±0.00	9.5±0.31	14.0±0.39	0.1±0.10	3.5±0.15	2.2±0.21	34.4±0.78	40.6±1.02
Deep Sea Bream (Blue Warehou)	Seriolella brama	0.7 ± 0.02	28.8±1.18	27.4±5.62	0.2±0.02	3.0±0.42	4.4±0.31	0.1±0.01	10.4±1.17	2.2±0.10	25.8±3.08	38.9±4.25
Deep Sea Cod (Ribaldo)	Mora moro	0.6 ± 0.01	32.0±0.09	10.8±0.38	0.1±0.01	2.8±0.06	3.9±0.18	0.1±0.02	4.6±0.14	1.2±0.02	47.1±0.46	53.1±0.58
Flake (Blue Shark)	Prionace glauca	1.3 ± 0.09	27.1±0.63	22.3±0.10	0.2±0.01	11.4±0.08	14.7±0.26	0.1±0.00	2.3±0.53	2.1±0.06	29.9±0.49	34.5±0.27
Flathead	Platycephalus bassensis	0.7 ± 0.02	36.0±0.26	12.0±0.86	0.1±0.01	4.0±0.04	5.5±0.02	0.1±0.02	3.2±0.05	1.1±0.04	41.5±0.69	46.1±0.64
Garfish	Hyporhamphus melanochir	0.8 ± 0.04	32.0±0.24	12.7±1.74	0.3±0.02	7.0±0.23	15.9±0.47	3.8±0.14	3.8±0.06	3.1±0.03	27.2±0.62	38.8±0.76
Gemfish	Rexen Solandri	6.3 ± 0.34	34.5±0.11	40.1±2.03	0.2±0.01	1.2±0.14	3.8±0.08	0.8±0.07	3.3±0.10	2.0±0.04	14.4±2.01	21.4±1.97
John Dory	Zeus faber	0.6 ± 0.06	34.1±0.22	9.2±0.34	0.1±0.01	4.3±0.07	6.9±0.04	0.1±0.00	2.8±0.04	1.1±0.04	45.1±0.48	49.3±0.45
Ling	Genypterus blacodes	0.5 ± 0.01	29.1±0.05	17.8±0.15	0.2±0.02	5.1±0.05	6.5±0.07	0.1±0.01	4.7±0.03	1.5±0.01	39.9±0.29	46.3±0.25
Ocean Trout	Oncorhynchus mykiss	4.5 ± 0.30	37.2±1.35	37.2±1.35	0.2±0.01	0.9±0.14	10.5±0.30	0.8±0.03	5.2±0.11	2.0±0.03	12.2±1.47	20.7±1.54
Prawn (shrimp)	Metapenaeus SPP	1.2 ± 0.19	32.4±0.20	20.9±1.30	0.2±0.00	0.1±0.02	14.2±0.62	0.3±0.02	13.4±0.61	1.4±0.02	15.4±0.20	30.7±0.61
Rainbow Trout	Oncorhynchus mykiss	1.7 ± 0.05	32.9±0.23	39.0±0.45	0.2±0.00	1.4±0.02	8.5±0.08	0.5±0.01	5.5±0.10	1.3±0.01	17.0±0.39	24.9±0.48
Red Snapper (Bight Red Fish)	Centroberyx gerrardi	0.7 ± 0.05	32.9±0.23	12.5±0.20	0.1±0.01	3.3±0.05	5.3±0.52	0.2±0.01	3.6±0.08	1.7±0.02	43.3±0.83	49.3±1.26
Australian Salmon	Arripis trutta	1.0 ± 0.06	34.0±0.17	9.1±0.19	0.2±0.02	4.8±0.07	7.4±0.15	0.3±0.00	5.7±0.09	4.1±0.01	38.2±0.18	48.6±0.26
Skate	Irolita Waitii	1.0 ± 0.13	38.5±0.21	15.1±0.27	0.3±0.00	13.3±0.09	17.7±0.12	0.1±0.01	3.1±0.05	2.4±0.02	22.0±0.07	27.8±0.05
Snook	Sphyraena novaehollandiae	1.5 ± 0.55	35.4±0.23	12.3±0.41	0.1±0.03	4.6±0.07	6.7±0.26	0.5±0.01	1.3±0.01	1.3±0.01	37.6±0.28	45.0±0.29
Squid	Sepioteuthis australis	1.1 ± 0.07	36.2±0.14	7.6±0.18	0.1±0.01	1.7±0.03	2.3±0.07	0.1±0.01	10.2±0.10	0.5±0.03	42.6±0.17	53.7±0.05
Swordfish	Xiphias gladius	14.4± 0.34	27.0±0.44	50.7±0.73	0.3±0.02	0.9±0.01	3.6±0.02	0.4±0.03	2.0±0.18	3.4±0.01	11.5±0.07	17.7±0.31
Tommy Ruff (Australian Herring)	Arripis georgianus	1.2 ± 0.08	36.0±0.21	17.6±1.14	0.2±0.01	3.5±0.16	6.2±0.10	0.5±0.07	5.2±0.13	1.9±0.05	29.3±0.32	39.4±1.14
Southern Bluefin Tuna	Thunnus maccoyii	0.7 ± 0.01	34.4±0.64	22.6±0.22	0.1±0.03	4.2±0.01	7.2±0.28	0.3±0.02	3.8±0.05	1.7±0.15	29.3±0.32	35.4±0.19
Northern Whiting	Sillago Sihama	1.0 ± 0.07	32.9±0.21	19.6±0.94	0.2±0.01	0.1±0.03	16.9±0.41	0.4±0.01	10.8±0.33	4.3±0.54	13.8±0.39	29.1±0.65

n–6: Omega 6, n–3: Omega 3. † Linoleic acid, ‡ Arachidonic acid, § Alpha linolenic acid, §§Eicospentaenoic acid, §Docosapentaenoic acid, †† Docosahexaenoic acid

Taken from Levels of Omega 3 fatty acids in Australian seafood. Sahar S A M Soltan PhD and Robert A Gibson PhD. *Asia Pac J Clin Nutr 2008;17 (3): 385-390.*

APPENDIX B. Amounts of omega-3 fatty acids in fish and amount of fish consumption required to provide 1g of omega-3 per day.

Name of Fish	Amount of Omega 3 FA (mg/100g)	Amount of Omega 3 FA (mg/150g Serve)	Amount of fish consumption required to Provide 1 g Omega 3 FA per day
Ling	222	334	450
Southern Bluefin Tuna	230	345	435
Blue Grenadier	247	370	405
Deep Sea Bream (Blue Warehou)	257	385	389
Coral Trout	270	408	370
Barramundi	276	415	362
Skate	289	433	346
Northern Whiting	302	455	313
John Dory	315	473	317
Boar	319	506	313
Garfish	327	489	306
Flathead	337	505	297
Deep Sea Cod (Ribaldo)	340	510	294
Red Snapper (Bight Redfish)	357	533	280
Prawn (shrimp)	373	562	268
Rainbow Trout	415	627	241
Flake (Blue Shark)	456	684	219
Australian Salmon	476	714	210
Tommy Ruff (Australian Herring)	477	716	210
Squid	584	874	171
Snook	675	1012	148
European Carp	829	1244	121
Ocean Trout	921	1380	108
Gemfish	1360	2033	74
Atlantic Salmon	2252	3380	44
Swordfish	2571	3860	39

Taken from Levels of Omega 3 fatty acids in Australian seafood. Sahar S A M Soltan PhD and Robert A Gibson PhD. *Asia Pac J Clin Nutr 2008;17 (3): 385-390*

APPENDIX C. SUPPLEMENTAL MATERIAL – Billman *et al.*, 2012. Red blood cell omega-3 polyunsaturated fatty acid content

	EPA		DHA		Omega-3 Index	
	Pre	Post	Pre	Post	Pre	Post
Placebo (n = 13)	0.16 ± 0.03	0.20 ± 0.03	0.25 ± 0.04	0.23 ± 0.04	0.40 ± 0.05	0.44 ± 0.06
1 g/day (n = 7)	0.23 ± 0.05	1.22 ± 0.14*[#]	0.17 ± 0.02	1.86 ± 0.17*[#]	0.40 ± 0.04	3.02 ± 0.23*[#]
2 g/day (n = 12)	0.16 ± 0.02	1.66 ± 0.12*[#]	0.27 ± 0.08	2.01 ± 0.13*[#]	0.55 ± 0.12	3.72 ± 0.22*[#]
4 g/day (n =29)	0.20 ± 0.01	3.91 ± 0.19*[#]	0.23 ± 0.02	2.83 ± 0.15*[#]	0.42 ± 0.03	6.76 ± 0.28*[#]

All values as are mean ± SE and are expressed as % total lipid content; EPA = eicosapentaenoic acid; DHA = docasahexaenoic acid; omega-3 index = EPA + DHA * P<0.01 Pre vs. Post; # = P<0.01 omega-3 dose vs. placebo.

ANOVA results

EPA: Dose $F_{3/57} = 84.79$, $P<10^{-6}$; Pre-post, $F_{1/57} = 196.67$, $P<0.10^{-6}$; Dose x Pre-post interaction, $F_{3/57} = 81.07$, $P<10^{-6}$

DHA: Dose $F_{3/57} = 52.64$, $P<10^{-6}$; Pre-post, $F_{1/57} = 228.30$, $P<0.10^{-6}$; Dose x Pre-post interaction, $F_{3/57} = 42.25$, $P<10^{-6}$

Omega-3 Index: Dose $F_{3/57} = 101.74$, $P<10^{-6}$; Pre-post, $F_{1/57} = 322.22$, $P<0.10^{-6}$; Dose x Pre-post interaction, $F_{3/57} = 91.27$, $P<10^{-6}$

APPENDIX D. SUPPLEMENTAL MATERIAL – Billman *et al.*, 2012.

Cardiac tissue omega-3 polyunsaturated fatty acid content

	EPA	DHA	Omega-3 Index
Right Atrium			
Placebo (n = 12)	0.22 ± 0.08	0.38 ± 0.11	0.58 ± 0.16
1 g/day (n = 7)	1.20 ± 0.10*	3.26 ± 0.21*	4.46 ± 0.22*
2 g/day (n = 12)	1.45 ± 0.23*	2.89 ± 0.41*	4.34 ± 0.64*
4 g/day (n =29)	2.10 ± 0.28*	2.71 ± 0.33*	4.80 ± 0.59*
Left ventricle			
Placebo (n = 12)	0.23 ± 0.04	0.36 ± 0.07	0.59 ± 0.09
1 g/day (n = 7)	1.22 ± 0.08*	2.92 ± 0.21*	4.14 ± 0.25*
2 g/day (n = 12)	1.69 ± 0.20*	2.67 ± 0.21*	4.35 ± 0.39*
4 g/day (n =29)	3.25 ± 0.24*	2.99 ± 0.17*	6.24 ± 0.39*

All values as are mean ± SE and are expressed as % total lipid content; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; omega-3 index = EPA + DHA
* $P<0.01$ omega-3 dose vs. placebo.

ANOVA results

Right Atrium:

EPA, $F_{3/56} = 7.83$, $P = 0.000188$; DHA $F_{3/56} = 9.51$, $P = 0.000036$;
Omega-3 Index: $F_{3/56} = 8.58$, $P = 0.000089$

Left Ventricle:

EPA, $F_{3/56} = 30.80$, $P<0.10^{-6}$; DHA, $F_{3/56} = 36.46$, $P<10^{-6}$;

Omega-3 Index, $F_{3/56} = 34.52$, $P<10^{-6}$

APPENDIX E. Human Donor Heart Phospholipid Fatty Acid Profile

	Left Atria	Left Ventricle	Right Atria	Right Ventricle
N	16	16	16	16
14:0	0.41±0.07	0.38±0.08	0.52±0.13	0.39±0.08
14:1n-7	0.03±0.03	0.006±0.02	0.00±0.00	0.002±0.00
15:0	0.09±0.07	0.11±0.05	0.10±0.08	0.10±0.05
15:1	0.08±0.14	0.02±0.08	0.01±0.03	0.03±0.07
16:0	17.53±1.43	14.46±0.60	15.64±2.87	15.18±0.81
16:1n-7	1.52±0.83	0.61±0.50	2.48±1.19	2.05±1.80
17:0	0.25±0.09	0.26±0.05	0.29±0.07	0.30±0.06
17:1n-7	0.40±0.39	0.31±0.49	0.05±0.12	0.02±0.04
18:0	16.15±1.70	15.76±1.60	16.78±2.27	16.55±1.32
18:1n-7	1.94±0.33	1.60±0.35	1.92±0.39	1.63±0.20
18:1n-9c	11.51±1.69	9.71±1.35	10.77±1.89	10.10±1.60
18:1n-9t	0.13±0.07	0.26±0.12	0.01±0.03	0.24±0.14
18:2n-6c	17.41±1.97	21.64±2.04	16.67±1.34	21.58±1.86
18:2n-6t	0.02±0.03	0.10±0.22	0.03±0.07	0.01±0.02
18:3n-3	0.13±0.07	0.21±0.07	0.08±0.08	0.02±0.04
18:3n-6	0.005±0.01	0.005±0.01	0.03±0.07	0.01±0.01
20:0	0.11±0.05	0.10±0.05	0.11±0.17	0.12±0.06
20:1n-9	0.30±0.24	0.27±0.08	0.31±0.22	0.38±0.24
20:2n-6	0.12±0.06	0.08±0.05	0.07±0.07	0.10±0.04
20:3n-3	0.05±0.04	0.05±0.04	0.02±0.04	0.03±0.04
20:3n-6	0.77±0.15	0.83±0.17	0.78±0.12	0.79±0.13
20:4n-6	21.54±1.62	24.44±2.13	22.52±1.91	22.20±2.36
20:5n-3	0.63±0.31	0.75±0.39	0.74±0.30	0.61±0.32
21:0	0.07±0.04	0.07±0.04	0.04±0.05	0.06±0.05
22:0	0.13±0.08	0.19±0.09	0.12±0.16	0.20±0.12
22:2n-6	0.01±0.03	0.02±0.04	0.06±0.14	0.01±0.03
22:4n-6	0.48±0.20	0.48±0.16	0.68±0.18	0.48±0.12
22:5n-3	1.98±0.45	1.94±0.43	2.15±0.41	1.75±0.46
22:5n-6	0.31±0.14	0.36±0.13	0.43±0.15	0.37±0.13
22:6n-3	5.17±1.51	4.42±1.23	6.12±2.08	4.06±1.13
24:0	0.12±0.02	0.12±0.01	0.11±0.03	0.13±0.01
24:1	0.37±0.04	0.29±0.03	0.34±0.05	0.30±0.03
ΣSFA	34.87±1.74	31.45±1.39	33.70±2.01	33.02±1.17
ΣMUFA	16.33±2.22	13.11±1.76	15.90±1.97	14.78±1.86
ΣPUFA	48.65±2.76	55.34±1.31	50.40±2.86	52.20±2.15
ΣPUFA n-6	40.68±2.37	47.97±1.68	41.28±2.10	45.58±1.87
ΣPUFA n-3	7.97±1.71	7.38±1.51	9.12±2.24	6.62±1.42
ΣEPA+DHA	5.81±1.74	5.17±1.56	6.86±2.33	4.67±1.38

Σ - sum of. Results are means±SD.

APPENDIX F. Human Explanted (Failing) Heart Phospholipid Fatty Acid Profile

	Left Atria	Left Ventricle	Right Atria	Right Ventricle
N	22	22	22	22
14:0	0.49±0.12	0.45±0.09	0.60±0.24	0.45±0.11
14:1n-7	0.004±0.02	0.01±0.02	0.00±0.00	0.02±0.04
15:0	0.11±0.08	0.11±0.07	0.14±0.09	0.11±0.06
15:1	0.06±0.10	0.05±0.18	0.02±0.05	0.02±0.06
16:0	18.00±1.21	15.58±1.42	16.41±2.38	16.32±1.33
16:1n-7	1.41±1.15	1.25±0.80	2.87±1.77	1.87±1.98
17:0	0.27±0.08	0.26±0.08	0.28±0.09	0.29±0.04
17:1n-7	0.31±0.65	0.13±0.15	0.05±0.13	0.10±0.18
18:0	16.68±1.82	17.10±1.58	15.92±1.75	17.02±1.29
18:1n-7	2.05±0.20	1.65±0.30	1.94±0.29	1.70±0.24
18:1n-9c	12.13±1.75	10.38±1.32	12.18±1.54	10.91±1.58
18:1n-9t	0.12±0.12	0.18±0.12	0.01±0.3	0.05±0.08
18:2n-6c	18.13±2.20	20.49±2.33	17.26±2.11	20.23±2.45
18:2n-6t	0.21±0.84	0.37±1.20	0.02±0.07	0.01±0.02
18:3n-3	0.20±0.08	0.22±0.09	0.19±0.10	0.22±0.07
18:3n-6	0.006±0.03	0.02±0.05	0.01±0.04	0.01±0.03
20:0	0.08±0.06	0.08±0.06	0.07±0.06	0.11±0.06
20:1n-9	0.40±0.29	0.21±0.13	0.32±0.35	0.28±0.31
20:2n-6	0.23±0.49	0.35±0.69	0.09±0.07	0.10±0.06
20:3n-3	0.02±0.04	0.05±0.06	0.02±0.04	0.02±0.03
20:3n-6	0.72±0.21	0.71±0.16	0.73±0.14	0.71±0.17
20:4n-6	19.25±2.45	21.70±3.10	20.41±2.48	20.95±2.82
20:5n-3	0.64±0.37	0.67±0.29	0.73±0.29	0.68±0.27
21:0	0.11±0.23	0.03±0.05	0.05±0.05	0.04±0.04
22:0	0.11±0.08	0.17±0.10	0.14±0.11	0.21±0.12
22:2n-6	0.00±0.00	0.01±0.02	0.05±0.14	0.02±0.04
22:4n-6	0.40±0.14	0.40±0.15	0.55±0.16	0.39±0.12
22:5n-3	1.53±0.43	1.63±0.38	1.79±0.33	1.55±0.36
22:5n-6	0.19±0.10	0.26±0.09	0.29±0.10	0.27±0.10
22:6n-3	5.47±1.80	4.97±1.59	6.33±1.92	4.77±1.56
24:0	0.09±0.02	0.10±0.01	0.14±0.02	0.15±0.02
24:1	0.40±0.03	0.35±0.02	0.39±0.05	0.40±0.04
ΣSFA	35.91±2.60	33.90±2.19	33.75±2.11	34.71±2.13
ΣMUFA	16.86±1.89	14.20±1.96	17.79±2.12	15.35±3.22
ΣPUFA	47.01±3.55	51.85±3.01	48.46±3.19	49.94±3.87
ΣPUFA n-6	39.14±2.83	44.32±3.49	39.40±3.27	42.70±3.29
ΣPUFA n-3	7.87±01.94	7.53±1.63	9.06±1.99	7.24±1.60
ΣEPA+DHA	6.12±2.03	5.63±1.74	7.06±2.11	5.46±01.71

Σ - sum of. Results are mean±SD.