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Cardiac physiology and clinical efficacy of dietary fish oil clarified through cellular mechanisms of omega-3 polyunsaturated fatty acids

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Abstract
Reduced cardiac mortality and morbidity have long been observed in association with omega-3 long chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish consumption, without clear physiological explanation. This review seeks to identify mechanisms of action based on evidence: of physiological effects, active components and effective intakes. Fish oil pleiotropic effects reveal actions that are either intrinsic: effects on cardiac function dependent upon membrane incorporation; or extrinsic: indirect cardiac effects through vascular disease. Extrinsic actions require EPA + DHA doses >3 g/day. Intrinsic effects derive from usual dietary intakes, /day and include improved myocardial oxygen efficiency, heart rate, nutritional preconditioning against ischaemic injury, arrhythmias and heart failure. Myocardial Na+ and K+ currents are non-selectively modulated by omega-3 and omega-6 PUFA to stabilise cells in vitro, but not by fish oil-induced membrane change. In contrast, cellular Ca2+ overload involved in ischaemic injury, arrhythmia and spontaneous pacemaker activity are modulated by both dietary fish oil and in vitro omega-3 LC-PUFA. A potential linking role of bioactive epoxy and hydroxy PUFA derivatives requires investigation. Omega-3 DHA predominates over EPA in population intake, is preferentially incorporated into myocardium and is selectively active in heart rate and arrhythmia modulation, but EPA predominates in clinical trials. Myocardial selectivity for DHA and independent intrinsic and extrinsic physiological mechanisms underpinning diverse clinical endpoints can explain some contradictory outcomes of clinical trials. Intrinsic modulation of intracellular Ca2+ handling provides a unifying physiologically plausible basis for intrinsic fish oil actions and insight to nutritional optimisation of cardiac function.

Keywords
Omega-3 polyunsaturated fatty acid, dietary fish oil, Docosahexaenoic acid DHA, fatty acid metabolites, oxygen uptake, calcium overload, ion channel, heart rate, sudden cardiac death, heart failure

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ABSTRACT:

Reduced cardiac mortality and morbidity have long been observed in association with omega-3 long chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fish consumption, without clear physiological explanation. This review seeks to identify mechanisms of action, based on evidence of physiological effects, active components and effective intakes.

Fish oil pleiotropic effects reveal actions that are either intrinsic: effects on cardiac function dependent upon membrane incorporation; or extrinsic: indirect cardiac effects through vascular disease. Extrinsic actions require EPA+DHA doses >3g.d^{-1}. Intrinsic effects derive from usual dietary intakes, <1g.d^{-1} and include improved myocardial oxygen-efficiency, heart rate, nutritional preconditioning against ischaemic injury, arrhythmias and heart failure. Myocardial Na^+ and K^+ currents are non-selectively modulated by omega-3 and omega-6 PUFA to stabilise cells *in vitro*, but not by fish oil-induced membrane change. In contrast, cellular Ca^{2+} overload involved in ischaemic injury, arrhythmia and spontaneous pacemaker activity are modulated by both dietary fish oil and *in vitro* omega-3 LC-PUFA. A potential linking role of bioactive epoxy and hydroxy PUFA derivatives requires investigation. Omega-3 DHA predominates over EPA in population intake, is preferentially incorporated into myocardium and is selectively active in heart rate and arrhythmia modulation, but EPA predominates in clinical trials.

Myocardial selectivity for DHA and independent intrinsic and extrinsic physiological mechanisms underpinning diverse clinical endpoints can explain some contradictory outcomes of clinical trials. Intrinsic modulation of intracellular Ca^{2+} handling provides a unifying physiologically plausible basis for intrinsic fish oil actions and insight to nutritional optimisation of cardiac function.

KEY WORDS:

omega-3 polyunsaturated fatty acid; dietary fish oil; docosahexaenoic acid DHA; fatty acid metabolites; oxygen uptake; calcium overload; ion channel; heart rate; sudden cardiac death; heart failure.

Abbreviations:

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
AA	arachidonic acid
ALA	alpha-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EET	epoxyeicosatrienoic acids
EPA	eicosapentaenoic acid
ETYA	eicosatetraynoic acid
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico
HDoHE	hydroxydocosahexaenoic acids
HETE	hydroxyeicosatetraenoic acids
HRV	heart rate variability
I_f	funny current
IP_3	inositol trisphosphate
I_{TO}	transient outward current
LA	linoleic acid
LC	long-chain
PUFA	polyunsaturated fatty acids

1. INTRODUCTION

Epidemiological studies consistently demonstrate cardiovascular health benefits of including fish in the diet, first revealed as a low rate of ischaemic heart disease in Greenland Inuit (Bang and Dyerberg 1972, 1980). Evidence from many subsequent observational studies supports a role for omega-3 polyunsaturated fatty acids (PUFA) contained in fish, not only in clinical prevention of heart disease death, but in promoting heart health. The earliest of these was a prospective cohort study in the Netherlands, which commenced in 1960 with 872 healthy men aged 40-59 y and after 20 y follow-up reported that men who ate fish were 50% less likely to die prematurely from coronary heart disease (Kromhout et al. 1985). The Physicians Health Study, of over 20,000 men initially free of heart disease, also showed that regular fish consumption was associated markedly reduced cardiovascular mortality over 11 years (Albert et al. 1998) and a 30% lower risk of developing heart failure over 30 years (Wilk et al. 2012). A review of the evidence presented as the recommendation of the American Heart Association in 2002, concluded that risk of coronary heart disease morbidity and mortality is reduced in persons regularly consuming omega-3 PUFA, especially the long chain PUFA (LC-PUFA) eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) derived from fish or fish oil (Kris-Etherton et al. 2002).

The overall conclusion endorsing fish oil fatty acids was based on epidemiological and clinical trials data gathered over several decades, stimulated by the seminal observations in the 1970s of low cardiovascular disease incidence in Greenland Inuit (Dyerberg et al. 1978). However, not all studies have been confirmatory and debate surrounds the role of omega-3 PUFA in cardiac physiology, cardiovascular health and disease prevention. While numerous meta-analyses of clinical research data support this conclusion (Bucher et al. 2002; Hooper et al. 2004; Mozaffarian et al. 2005b; Zhao et al. 2009; Mozaffarian and Wu 2011; Musa-

Veloso et al. 2011; Casula et al. 2013), others do not (Hooper et al. 2006; Hooper et al. 2009; Khoueiry et al. 2013), creating controversy and argument.

Some clues to the both origins and a solution to the debate come from early observations reporting physiological effects of fish oil on the heart to prevent ventricular fibrillation or sustained arrhythmias during ischaemia and reperfusion in rats (McLennan et al. 1988), subsequently confirmed in many animal studies (Matthan et al. 2005; McLennan and Abeywardena 2005). This stimulated applied clinical interest, and a case control study of sudden arrhythmic death victims revealed that regular fish consumption was associated with reduced risk of fatal cardiac events, by virtue of the omega-3 LC-PUFA in the fish (Siscovick et al. 1995). Reviews and health authority reports cite a disparate range of potential mechanisms of action (Schmidt et al. 2000; Kris-Etherton et al. 2002; Schmidt et al. 2005; Colquhoun et al. 2008; Mozaffarian and Wu 2011; Baum et al. 2012). It is the premise of this review that much of the controversy surrounding omega-3 cardioprotection can be attributed to an inadequate understanding of the role of these fatty acids in cardiac physiology, which has resulted in clinical trials that are based on ill-defined or poorly constructed hypotheses and with inappropriate pooling of disparate population groups. For example, a clinical trial that found no effect of omega-3 LC-PUFA in reducing the risk of the "first major cardiovascular event", recruited equally from high risk myocardial infarction and stroke victims (Galan et al. 2011). It combined as the primary endpoint: fatal or non-fatal myocardial infarction; sudden arrhythmic death; fatal or nonfatal stroke; aortic dissection; and heart failure. The primary endpoint therefore considered a variety of disparate physiological mechanisms of action of omega-3 PUFA, such as prevention of arrhythmia, thrombosis, atherosclerosis, infarction, hypertension, vascular medial damage and heart failure as being equally valid. A stroke victim is not necessarily at risk of heart failure or myocardial infarction.

The failure of some clinical intervention studies to fulfil the promise of the epidemiology may also emanate from the common use of fish oil supplements in clinical trials that do not represent the omega-3 fatty acids consumed in fish. Again, this discrepancy is based on incomplete understanding of the mechanisms of action of fish oil fatty acids and imprecise recognition of the active component(s). With little understood about the underlying mechanisms of n-3 PUFA effects on cardiac function and dysfunction, there is "ambivalence to adoption and incorporation into guidelines" (Tang and Samara 2011). A widespread misalignment of hypotheses with known mechanisms of action will be explained through the evidence for an intrinsic role of fish oil fatty acids in cardiac physiology, then integrated with the evidence for and against individual cellular and ionic mechanisms to develop a unifying hypothesis.

1.1. Omega-3 polyunsaturated fatty acids

Polyunsaturated fatty acids cannot be formed de novo in mammals and must be obtained from the diet. The principal omega-6 PUFA found incorporated into cardiac membranes are linoleic acid (18:2 n-6) and arachidonic acid (AA, 20:4 n-6) (numbering derived from 20 carbon chain with 4 double bonds, with the first at the 6^{th} carbon from the methyl terminal of the molecule). Intake of omega-6 fatty acids is widespread in western diets, with linoleic acid readily obtained from plants, especially grains and seed oils (Harris et al. 2009b). Whilst some arachidonic acid is obtained from meat, most is formed from linoleic acid, which is readily converted to arachidonic acid in the body and there is no inter-conversion of omega-6 to omega-3 PUFA families (Sprecher 2000) (Figure 1). Some dietary plant sources such as linseed (flax), canola and soy contain the omega-3 PUFA α-linolenic acid (ALA, 18:3 n-3), however substrate competition between omega-3 PUFA and omega-6 PUFA occurs at all steps in the pathway of elongation and desaturation (Figure 1), especially at the rate limiting

δ-6 desaturase enzyme. With this competition, together with the preponderance of omega-6 PUFA in the western diet, formation of DHA from its plant precursor is extremely poor (Sprecher 2000; Burdge and Calder 2005). Moreover, the δ-6 desaturase enzyme is involved at two points in the pathway and as ALA increases from an optimal concentration, the total dietary PUFA concentration (omega-3 plus omega-6) becomes inhibitory to the production of DHA (Figure 1) (Gibson et al. 2013). As a consequence, DHA concentrations in human tissues actually decline with high dietary intakes of ALA (Burdge and Calder 2005). Therefore the tissue concentrations of EPA and especially DHA can be best elevated by direct dietary intake of fish or fish oils containing the preformed omega-3 PUFA (Metcalf et al. 2007; Harris et al. 2009a; Slee et al. 2010). It has been suggested that dietary linoleic acid intake should be reduced to restore the balance (Simopoulos 2006), whilst others argue that omega-6 PUFA and omega-3 PUFA are equally vital to human health (Harris 2010). A debate about the importance of dietary balance of omega-3/omega-6 PUFA has established that the ratio, whilst important in determining the fate of dietary ALA (Harris 2006; Gibson et al. 2013) has no influence on membrane incorporation when omega-3 fatty acids are consumed as the LC-PUFA EPA or DHA (Griffin 2008; Slee et al. 2010) and also has no influence on the clinical efficacy of fish oil fatty acids (Mozaffarian et al. 2005a; Harris et al. 2009b). This debate has been further clouded by failure to be explicit about which of the omega-3 PUFA have been investigated (Abbott et al. 2012). Therefore, for the present review, the term LC-PUFA is used to define only those fatty acids of 20 carbons or longer (e.g. fish oil fatty acids EPA and DHA, not ALA).

[Figure 1. near here]

Early observations of high bleeding tendency in Greenland Inuit associated with the change in balance of enzymic production of the pro-aggregation vasoconstrictor eicosanoid thromboxane and anti-aggregatory vasodilator eicosanoid, prostacyclin from EPA in platelets and endothelium (Dyerberg et al. 1978), together with demonstration of triacylglycerol

lowering in clinical settings (Connor et al. 1993) and blood pressure lowering or antihypertensive actions (Morris et al. 1993), alluded to several potential physiological roles for the omega-3 fatty acids. However, it is now recognised that high daily intakes are necessary to inhibit haemostasis and thrombosis or reduce triacylglycerols or blood pressure in human studies (Morris et al. 1993; Nordoy et al. 2001; Cabo et al. 2012; Wachira et al. 2014). (Daily intakes of 6 g omega-3 LC-PUFA as fish oil had no significant effect on post-surgical bleeding after coronary artery bypass graft or valve surgery (Metcalf et al. 2007); fish oil and concentrates of >3 $g.d^{-1}$ omega-3 LC-PUFA are used therapeutically to lower plasma triacylglycerols effectively (Skulas-Ray et al. 2011); small reductions in BP ranging from 1 mmHg to 6 mmHg are achieved with daily 2-4 g of omega-3 LC-PUFA, and clinically-relevant BP changes require more than 3 $g.d^{-1}$ of omega-3 LC-PUFA from fish or fish oil (Cabo et al. 2012)). Contrast these high intakes with those associated with the reduced risk of sudden cardiac death (regular intake of fish equivalent to omega-3 LC-PUFA intake of 100 $mg.d^{-1}$ is associated with 30% reduction of risk and 185 $mg.d^{-1}$ is associated with 50% reduction in risk (Siscovick et al. 1995)), or prevention of heart disease deaths (15% reduction in mortality with each 100 $mg.d^{-1}$ intake of omega-3 LC-PUFA from fish or fish oil with no further benefit beyond 250 $mg.d^{-1}$ (Mozaffarian and Rimm 2006)). Moreover, secondary prevention of heart disease deaths has been achieved in clinical trials with low intakes of fish oil omega-3 fatty acids (<1$g.d^{-1}$) despite little change in the classical cardiovascular risk factors of blood pressure or triacylglycerols (Burr et al. 1989; Valagussa et al. 1999; Tavazzi et al. 2008). The large prospective cohort studies: the Physicians Health study (Albert et al. 1998); and the Zutphen Study (Kromhout et al. 1985; Streppel et al. 2008), both observed the primary prevention of cardiovascular deaths with 1-2 fish meals per week, and found that the omega-3 LC-PUFA intake was associated with reduced risk of sudden arrhythmic death. This reduced risk of sudden death was, on the one hand, not associated with reduced risk of myocardial infarction (Albert et al. 1998), and on the other

hand, unlikely to be associated with effects on lipids and lipoprotein metabolism, platelet aggregation or bleeding time (Kromhout et al. 1985). An alternative mechanism of action to platelet function, blood pressure regulation or blood lipids and atherosclerosis must be sought to fully explain the consistent cardiovascular benefits associated with the moderate consumption of fish.

2. A CARDIAC BASIS FOR OMEGA-3 ACTIONS

2.1. Cardiac Arrhythmia

In both the general population and in high risk cardiac patients, consumption of omega-3 LC-PUFA is associated with fewer cardiac deaths. There is a particularly high risk of sudden arrhythmic death in the first 5 years after a heart attack (myocardial infarction), making this an ideal target population for studying prevention of fatal arrhythmias. The Diet and Reinfarction Trial, a secondary prevention trial of 2033 men who had recovered from a myocardial infarction, reported reduced cardiovascular mortality with regular intake of 2-3 portions of fatty fish per week (Burr et al. 1989), which because there was no significant difference in new ischaemic events, was indicative of the prevention of arrhythmic sudden death. Similarly, a large secondary prevention trial of more than 11,000 high-risk patients post-myocardial infarction (GISSI Prevenzione trial), used the specific endpoint of sudden death and demonstrated lower mortality rates associated with 850 $mg.d^{-1}$ of EPA+DHA in capsule form (Valagussa et al. 1999). In these large clinical trials, the mortality benefit was achieved without reduction in new ischaemic events (i.e. no fewer new heart attacks but fewer deaths). In the GISSI Prevenzione trial the reduction in risk of sudden cardiac death was evident within 120 days of starting supplementation, compared with double that time for mortality from other cardiovascular causes (Marchioli et al. 2002). Together with epidemiological evidence of the primary prevention of sudden death with regular fish consumption in case-controlled (Siscovick et al. 1995) and cohort (Albert et al. 1998;

Streppel et al. 2008) observational studies, the mechanisms of benefits of modest intakes of fish oil fatty acids have diverged from effects on plasma lipids, vascular disease and ischaemic events that represent one grouping of mechanisms of action (Kromhout et al. 2012). Other possible mechanisms however, converge with a large body of physiological evidence from animal dietary studies demonstrating prevention of heart failure (Duda et al. 2007; McLennan et al. 2012), and *in vivo* antiarrhythmic effects in several species, including: rat (Hartog et al. 1987; McLennan et al. 1988; Gudbjarnason 1989; McLennan et al. 1989a; Hock et al. 1990; McLennan et al. 1990; McLennan 1993; al Makdessi et al. 1995); pig (Hartog et al. 1987); and marmoset monkey (McLennan et al. 1992b, 1993). Furthermore, the *in vitro* perfused heart studies illustrate the direct intra-cardiac (intrinsic) arrhythmia and heart failure prevention and heart rate slowing are not dependent upon circulating fatty acids (Pepe and McLennan 1996; Abdukeyum et al. 2008; McLennan et al. 2012).

2.1.1. Heart Rate variability

Physiological evidence supporting the antiarrhythmic action of fish oil fatty acids is also derived from the association of omega-3 fatty acid consumption with increased heart rate variability (HRV) (Christensen et al. 1999; Christensen et al. 2001a; Christensen et al. 2001b; Villa et al. 2002) (Xin et al. 2013) and with lower heart rate (Vandongen et al. 1993; Christensen et al. 1999; Mozaffarian et al. 2005c).

Heart rate variability applies a variety of metrics to describe the oscillations in heart period of consecutive beats, and the oscillations of heart rate over time (Camm et al. 1996; Kleiger et al. 2005). Physical activity and emotion have well known influences on heart rate, however even at rest, with a settled mean heart rate, the heart period fluctuates from beat to beat due to a dynamic interplay between multiple physiologic control mechanisms, providing fine tuning to cardiac output and mean arterial blood pressure. Short term regulation is under the constant

influence of parasympathetic and sympathetic neural activity, and other neurohormonal and circadian rhythms modulate heart rate over time. The major HRV metrics can be categorised within time and frequency domains (Kleiger et al. 2005). Importantly from an applied physiology perspective, high HRV is associated with exercise training, fitness and health, whereas low HRV is associated with stress, fatigue, heart failure and is proposed as a marker of risk of sudden arrhythmic death (Bilchick et al. 2002), being strongly associated with sudden death in post-myocardial infarction cardiac patients. Various high cardiac risk patient groups (chronic renal failure, diabetes mellitus type I, or patients with coronary artery disease), exhibit low HRV, which is modulated in association with omega-3 LC-PUFA from the diet (Christensen et al. 2001a; Christensen et al. 2001b; Villa et al. 2002; Carney et al. 2010; Christensen et al. 2010). Post-myocardial infarction patients also exhibit increased HRV after dietary intervention with marine lipids (Christensen et al. 1996; Christensen et al. 1997; O'Keefe et al. 2006; Mozaffarian et al. 2008) as do healthy men (Christensen et al. 1999). In a large cohort of over 5000 men and women aged >65 y, habitual fish consumption, providing omega-3 LC-PUFA intake over a normal dietary range (300-500 $mg.d^{-1}$) and higher, was associated with improved HRV, particularly in parameters indicating augmented vagal activity and reduced erratic sinus node firing (Mozaffarian et al. 2008). A systematic review and meta-analysis of nine separate fish oil intervention studies identified a significant increase in high frequency power indicative of increased vagal tone (Xin et al. 2013). In many respects, the omega-3 LC-PUFA appear to reduce heart disease risk similarly to regular exercise (Brown and Moore 2007; Marini et al. 2007; Powers et al. 2008) and improved HRV is another factor held in common between dietary fish oil and exercise training (Amano et al. 2001; Nunan et al. 2010; Grant and van Rensburg 2013).

2.1.2. Intrinsic Heart Rate

Physiologically, slowing the heart rate increases ventricular filling time and allows myocardial stretch to improve ejection force with less energy cost than sympathetic stimulation, improving cardiac efficiency. Clinically, this principle is utilised through the use of β-blockers in treatment of hypertension and heart failure. A change in heart rate has little impact on early rapid filling or late atrial filling but correlates very closely with diastasis, the middle phase of ventricular filling (Chung et al. 2004). Coronary blood flow is greatest in this period of diastole and slower heart rate has implications for coronary perfusion reserve and more efficient myocardial perfusion. Conversely, a high resting heart rate is a marker of elevated risk for cardiovascular mortality, particularly sudden death and heart failure (Kannel et al. 1987; Fox et al. 2007). Regular omega-3 LC-PUFA intake can lower resting heart rate by up to 5 beats per minute in patient and healthy populations (Grimsgaard et al. 1998; Mori et al. 1999; Dallongeville et al. 2003; Geelen et al. 2005; O'Keefe et al. 2006), in healthy individuals during exercise without compromising maximum heart rate (Peoples et al. 2008), and during exercise recovery in post-myocardial infarction patients (O'Keefe et al. 2006). Meta-analysis of randomised control trials with heart rate recorded as a secondary outcome found an average reduction of 2.5 beats per minute with fish oil supplementation (Mozaffarian et al. 2005c). Fish oil dietary-induced heart rate slowing is exhibited across many animal species, including: horse (O'Connor et al. 2004); pig (Hartog et al. 1987); rabbit (Verkerk et al. 2009a); and dog (Billman and Harris 2011), which together with the human evidence and a non-significant trend in marmoset monkey (McLennan et al. 1992a), provides evidence for a universal heart rate lowering effect of dietary omega-3 LC-PUFA (McLennan and Abeywardena 2005). The omega-3 LC-PUFA and exercise training share the ability to reduce resting heart rate. While heart rate and heart rate variability largely reflect the activity and balance of the autonomic nervous system, an intrinsic effect within the myocardium is evident from the ability of chronic fish oil feeding to reduce heart rate in human cardiac transplant patients (Harris et al. 2006); in rat isolated heart (Abdukeyum et al. 2008); and in

rabbit isolated pacemaker cells (Verkerk et al. 2009a), which in the absence of neural input illustrates an independence from autonomic control that is similarly demonstrable for arrhythmia prevention (Yang et al. 1993; Pepe and McLennan 1996; Abdukeyum et al. 2008).

Automaticity due to change in pacemaker potential during diastolic depolarisation is primarily ascribed to two ionic mechanisms: the pacemaker current attributed to channels permeable to both Na^+ and K^+ (funny current (I_f)); or to Ca^{2+} transients, most recently ascribed to intracellular Ca^{2+} cycling (Vinogradova and Lakatta 2009; Monfredi et al. 2013). The slowed heart rate *in vitro* in rabbits after dietary fish oil modulation has been partly explained by reduced I_f density and pacemaker activity in sinoatrial cells, however there remains residual activity, which implicates other membrane currents in the omega-3 LC-PUFA effects (Verkerk et al. 2009a). To date, the effects of omega-3 LC-PUFA on other specific ion channels in pacemaker cells have not been studied, however omega-3 LC-PUFA do influence a number of ion channels in non-pacemaker cardiac muscle cells and other cell types (McLennan 2004; Xiao et al. 2005) which are further considered below.

2.2. Cardiac function in healthy and failing heart

In addition to effects mediated through altered physiology of cardiac excitability and spontaneous depolarisation rate, clinical and experimental studies reveal prevention of heart failure as a further potential mechanism of fish oil action. A large epidemiology study of 4,738 adults aged over 65 y that showed 20% reduced incidence of heart failure associated with fish meals delivering estimated intakes of omega-3 LC-PUFA as low as 260 $mg.d^{-1}$ (Mozaffarian et al. 2005b), directly implicates effects on heart function whilst indirect evidence can be found in the reduced non-arrhythmic but otherwise unspecified cardiac deaths and post-infarction deaths (Valagussa et al. 1999). More recently, a randomised controlled trial in 6,975 patients with heart failure showed that 850 $mg.d^{-1}$ of EPA+DHA

reduced mortality and cardiac cause hospitalisations, with specific improvement in the proportion of subjects with low (<40%) ejection fraction (Tavazzi et al. 2008). Furthermore, a large cohort trial in general practice recruited 12,500 patients with multiple cardiovascular disease risk factors, and reported a 35% reduction in hospitalisations from heart failure as the only physiologically and clinically meaningful outcome from treatment with 850 $mg.d^{-1}$ EPA+DHA over 5 years (Risk and Prevention Study Collaborative Group 2013). These outcomes reflect improved myocardial function in clinical populations, and as with sudden cardiac death, they occur independently of dietary omega-3 LC-PUFA effects on classic risk factors such as plasma triacylglycerols and thrombogenic factors (Nordoy et al. 2001). Again, physiological roles within cardiac membranes are implicated. Animal studies describe fish oil-induced improvements in myocardial inotropy, lusitropy and efficiency of oxygen use by the myocardium that are associated with the incorporation of the omega-3 LC-PUFA DHA into myocardial membranes (McLennan et al. 1992a; Pepe and McLennan 2002, 2007; Abdukeyum et al. 2008). These have been translated into direct demonstration of improved contractility and sustained cardiac output in hypertrophy models of heart failure (Duda et al. 2007; McLennan et al. 2012) .

2.3. Membrane composition as a basis for omega-3 fatty acids cardiac effects.

In the laboratory rat, dietary fish oil (EPA+DHA: 1.75-4% of diet by weight or 3.5-8% of diet energy) produces marked changes in myocardial membrane phospholipid fatty acid composition, increasing the content of omega-3 LC-PUFA especially DHA and reducing the content of omega-6 PUFA arachidonic acid and linoleic acid (Charnock et al. 1986; Hartog et al. 1986; Hartog et al. 1987; McLennan et al. 1993; al Makdessi et al. 1995; McLennan et al. 1996; Pepe and McLennan 1996; McLennan 2001). The intakes employed historically are mostly unachievable in the equivalent human diet, raising questions of their relvance to human physiology. It was more recently established that maximal omega-3 LC-PUFA

incorporation is derived from smaller dietary intakes (EPA+DHA: 0.5-0.75% by weight or 1-1.5% energy) (McLennan et al. 1996; Owen et al. 2004). Notably from an applied physiology perspective, very small intakes in the rat (EPA+DHA: 0.05-0.2% by weight or 0.1-0.5% energy) can double to triple the myocardial membrane omega-3 LC-PUFA content almost to maximal incorporation, and this translates to 280 – 1200 $mg.d^{-1}$ for human consumption (Slee et al. 2010). Thus, physiological outcomes in the rat, can now be regarded as consistent with those observed in human populations, having dietary fish intakes associated with: reduced risk of sudden cardiac death (EPA+DHA: 100-450 $mg.d^{-1}$ (Siscovick et al. 1995); EPA+DHA: 250 $mg.d^{-1}$ (Mozaffarian and Rimm 2006)); reduced risk of incident heart failure (EPA+DHA: 250 $mg.d^{-1}$ (Mozaffarian et al. 2005b); EPA+DHA: 100 – 400 $mg.d^{-1}$ (Wilk et al. 2012)); and lower heart rates (fish more than once per week: EPA+DHA: 250 - >500 $mg.d^{-1}$ (Mozaffarian et al. 2006); fish more than once per week (Dallongeville et al. 2003)). It is likely therefore, that the omega-3 fatty acids exert their protective effects by their role in the fabric of the heart, affecting intrinsic myocardial physiology (McLennan et al. 2007) to modify sinoatrial node function, myocardial depolarisation and electrical stability, contractility and relaxation (Figure 2). Additional effects on risk factors of plasma lipids and blood pressure occur only at higher (pharmacological or therapeutic) intakes in excess of EPA+DHA 3 $g.d^{-1}$ (Skulas-Ray et al. 2011; Cabo et al. 2012) (Figure 2).

[Figure 2 near here]

2.4. Ongoing controversy on cardiovascular disease protection by fish oil fatty acids.

Not all reviews of the evidence determine a cardioprotective role of fish oil fatty acids. A comprehensive systematic review and meta-analysis raised some doubts about fish oil cardiovascular protection (Hooper et al. 2006; Hooper et al. 2009), but becomes quite revealing on sub-analysis to unpick the pooled clinical endpoints. With some endpoints,

effects seen in cohort studies were not reproduced in randomised controlled trials and these differences might be explained by the different sources of omega-3 LC-PUFA derived from the diet as opposed to supplements. While it is well established that food fish typically contain DHA in excess of EPA (Nichols et al. 1998; Kris-Etherton et al. 2002; Mozaffarian and Wu 2011), this has largely been ignored in designing clinical trials, where fish oil supplements predominantly provide EPA in excess of DHA. Furthermore, the cardiovascular events, of stroke, non-fatal myocardial infarction and vascular restenosis, which show less or no trend towards favouring fish oil can be attributed to vascular and lipid metabolism, whereas the cardiovascular events of fatal myocardial infarction, heart failure, and sudden cardiac death not only show trends in randomised control trials but significant effects of cohort studies on meta-analysis (Hooper et al. 2009; Xin et al. 2012) and can be attributed to intrinsic effects within the heart (Figure 2).

Why is this important? Sudden cardiac death accounts for many thousands of deaths annually in developed nations. Transient changes such as myocardial ischemia, or electrolyte imbalance can precipitate ventricular fibrillation (Myerburg et al. 1989). A great proportion of ischaemic episodes progress to myocardial infarction, which produces a vulnerable myocardial substrate, waiting for a trigger event for lethal arrhythmias to occur. The basis for subtle differences in vulnerability that may predispose the sudden death victim to fatal arrhythmia is not well understood (Huikuri et al. 2003), however experimental observations in acute ischaemia (McLennan et al. 1993), human intervention trial in patients with an existing myocardial infarction (Marchioli et al. 2002), and a case control study of primary cardiac arrest (Siscovick et al. 1995) suggests that omega-3 LC-PUFA stabilise the vulnerable myocardium and reduce the risk of the trigger causing arrhythmia. The GISSI Prevenzione trial recruited patients within two to three weeks of their index myocardial infarction and found the increased vulnerability amenable to omega-3 LC-PUFA was most

evident early, with 57% of the mortality benefit achieved within 90 d (Marchioli et al. 2002). Clinical trials such as the SU.FOL.OM3 trial, which investigated post myocardial infarction patients (potential vulnerable myocardial substrate) pooled together with those more at risk of vascular occlusion and ischaemic stroke (Galan et al. 2011) are likely to be underpowered to detect intrinsic cardiac mechanisms of omega-3 PUFA action and prevention of sudden cardiac death. By recruiting patients up to 12 months after their index event (median 101 d) the acute vulnerable period identified in GISSI Prevenzione may have also been missed.

3. SELECTIVITY AND SPECIFICITY OF OMEGA-3 EFFECTS

The cardiovascular protective effects of fish oil are not reproducible with dietary ALA as the source of omega-3 PUFA (Mozaffarian 2005; Mozaffarian and Wu 2011; Vedtofte et al. 2011). This confirms both the poor conversion of ALA to DHA (Brenna et al. 2009) and reflects the low presence of circulating ALA in plasma phospholipid (Gibson et al. 2013) and the extremely low concentration or absence of ALA in human (Sexton et al. 1995) or animal (McLennan and Dallimore 1995) cardiac tissue samples. Human studies do not readily afford biopsy of heart muscle to correlate composition with fish oil or omega-3 fatty acid consumption. Numerous animal studies report that DHA is the most abundant omega-3 fatty acid found in myocardial membrane phospholipids, with EPA often undetectable in rat heart unless supplemented with fish oil (Hartog et al. 1986; Gudbjarnason 1989; Hock et al. 1990; Woodcock et al. 1995; Pepe and McLennan 1996; Honen and Saint 2002; Pepe and McLennan 2002; Arnold et al. 2010; Slee et al. 2010). Similarly, marmoset monkey (Charnock et al. 1989; Charnock et al. 1992) and pig (Nair et al. 2000; Coronel et al. 2007) display higher myocardial concentrations of DHA than EPA prior to fish oil feeding. Fish and fish oil contain both EPA and DHA, however fish oil feeding in the rat favours myocardial enrichment with DHA. This occurs irrespective of whether a high EPA fish oil (Hartog et al. 1986; Gudbjarnason 1989; Hock et al. 1990; Woodcock et al. 1995; Pepe and

McLennan 1996; Honen and Saint 2002; Pepe and McLennan 2002; Arnold et al. 2010) or a DHA rich fish oil is consumed, at high (5-12% of diet by weight) (Charnock et al. 1986; McLennan et al. 1993; McLennan 2001) or low (0.16-1.25%) intakes (Slee et al. 2010). Similar excess of DHA over EPA is found in the myocardium of a non-human primate the marmoset monkey after fish oil feeding (Charnock et al. 1992). In contrast, pigs fed a high EPA fish oil or equal concentrations of EPA and DHA show markedly greater myocardial enrichment of EPA than DHA (Nair et al. 1999; Nair et al. 2000; Coronel et al. 2007). Similar to the pig, dog hearts are peculiarly resistant to incorporation of DHA (Judé et al. 2007; Billman et al. 2010). Dog ventricle shows greater enrichment with EPA than DHA after 12 w of 4 $g.d^{-1}$ fish oil ethyl ester concentrate, delivering 3360 $mg.d^{-1}$ EPA+DHA to 20 kg dogs (Billman et al. 2010) or even after feeding a high DHA dietary supplement delivering 456 $mg.d^{-1}$ EPA+DHA for 8 w (Judé et al. 2007). However in the rat, even feeding a diet enriched with purified EPA produces a greater increase in membrane phospholipid DHA content with little incorporation of EPA (McLennan et al. 1996; McLennan and Raederstorff 1999). This contrasts with the direct incubation of isolated myocytes with EPA which results in EPA enrichment of the membrane fatty acids (deJonge et al. 1996a). In dietary studies the enrichment of myocardial DHA even occurs against a background of high circulating total plasma EPA in the rat (McLennan 2001) and with high or low background omega-6 PUFA (Slee et al. 2010).

Whilst few in number, analyses of both human ventricle and human atria consistently report DHA incorporation at markedly higher concentrations than EPA (Rocquelin et al. 1985; Sexton et al. 1995; Garg et al. 2006; Metcalf et al. 2007; Metcalf et al. 2010). Atria, which are the most often sampled human myocardial tissue, sustain DHA enrichment well above EPA after fish oil supplementation, with concentrations of DHA reaching 8-10% compared

with maximum of 2-4% EPA (Garg et al. 2006; Metcalf et al. 2007; Metcalf et al. 2010)}, implicating a primary role for DHA in human cardiac physiology, as in the rat.

In contrast to myocardium, detailed fatty acid composition is more readily available from human plasma, red blood cell and skeletal muscle. Rat and marmoset monkey studies report that the composition of skeletal muscle phospholipid fatty acids closely resemble heart, having high DHA and low EPA concentrations irrespective of the background diet (Charnock et al. 1989; Charnock et al. 1992; Helge et al. 1999) and respond to fish oil feeding by increasing DHA incorporation (Peoples and McLennan 2010). Similarly, human adult or infant skeletal muscle also incorporates more DHA than EPA under a variety of conditions (Borkman et al. 1993; Baur et al. 2000; Di Marino et al. 2000; Helge et al. 2001). Holub and co-workers, in a series of studies of Canadian Inuit, Cree, and residents of Quebec and Guelph found that irrespective of fish intake, DHA exceeds EPA in plasma phospholipids (Dewailly et al. 2001a; Dewailly et al. 2001b; Conquer et al. 2002; Dewailly et al. 2002) even when relative intakes of EPA and DHA are very similar (Dewailly et al. 2001a). Finally, red blood cell fatty acid composition and the concentration sum of EPA+DHA has been proposed as a risk factor for cardiovascular disease, the omega-3 index (Harris and von Schacky 2004), acting as a biomarker of omega-3 PUFA status that correlates well with myocardial omega-3 LC-PUFA content in man (Metcalf et al. 2010) and rats (Owen et al. 2004; Stark 2008). Dietary supplementation of healthy young adults for five months with fish oil containing EPA in excess of DHA, resulted in dose related increases in red blood cell omega-3 index (Flock et al. 2013b). At all doses of fish oil, up to 1800 $mg.d^{-1}$, despite an excess of EPA over DHA, the red blood cell membrane concentration of DHA increased more, and always exceeded that of EPA (even with placebo), confirming many other reports of higher DHA than EPA concentrations in human red blood cells (Stark 2008; Metcalf et al. 2010; Flock et al. 2013a; Patterson et al. 2014). Dietary supplementation of animals and

humans also demonstrates selectivity of DHA over EPA for lowering heart rate (Grimsgaard et al. 1998), lowering blood pressure and improving vascular reactivity (enhancing vasodilator and attenuating vasoconstrictor responses) (McLennan et al. 1996; Mori et al. 1999; Mori et al. 2000). While these studies suggest a small negative association between plasma lipid risk factors (HDL/ total cholesterol, triacylglycerols) and omega-3 LC-PUFA that appear to be slightly stronger for EPA than DHA, there is often little change in these factors at doses associated with the reduced incidence of sudden cardiac death (Burr et al. 1989; Siscovick et al. 1995; Albert et al. 1998; Valagussa et al. 1999; Marchioli et al. 2002; Lemaitre et al. 2003). Contrast that with the JELIS study (Yokoyama et al. 2007), which delivered 1800 $mg.d^{-1}$ of pure EPA supplement to hypercholesterolaemic patients and identified significant reduction in vascular / lipid related events but no difference in sudden cardiac death, implicating EPA in extrinsic but not intrinsic effects of fish oil.

4. ELECTROPHYSIOLOGICAL EFFECTS OF OMEGA-3 FATTY ACIDS.

When considering cellular mechanisms underpinning the effects of dietary inclusion of fish oil on cardiac function and risk, there are two primary considerations:

- Dietary fish deliver DHA as the principal omega-3 fatty acid.
- The human myocardium selectively incorporates DHA over EPA.

From these observations, two conditions should be met in interpreting *in vitro* and *in vivo* studies of physiological mechanisms in order to explain the association of omega-3 LC-PUFA consumption with clinical outcomes in population and dietary intervention studies.

- Effects of acutely administered fatty acids on ion channels or intracellular mechanisms must be reproducible with dietary intervention and membrane incorporation.
- Effects attributable to the omega-3 PUFA must be specific and not reproduced by omega-6 PUFA, solvents or detergents.

4.1. Whole animal:

In order for the putative intrinsic antiarrhythmic and cardiac slowing actions of dietary omega-3 LC-PUFA to be fully accepted, a physiologically plausible mechanism of action needs to be substantiated (Tang and Samara 2011). In particular, for a sound basis in physiology nutritional effects must be distinguished from a selective acute therapeutic effect or a laboratory curiosity (i.e. can effects obtained from acute ingestion replicate effects of habitual consumption). Acute exposure to omega-3 fatty acids prevents cardiac arrhythmias *in vivo* and dysrhythmias of isolated myocytes *in vitro*. Ventricular fibrillation is inducible on demand in dogs with an existing myocardial infarction by acute coronary occlusion while they are exercising (Billman et al. 1994; Billman et al. 1997). This is a reliable and reproducible stimulus for arrhythmias which can be tested and re-tested between treatments and in recovery. The combined stress of acute coronary artery occlusion and exercise (ischaemia plus sympathetic stimulation) induces arrhythmias which are prevented by acute infusion of fish oil fatty acid emulsions (Billman et al. 1994; Billman et al. 1997). In this post-infarction model of sudden death, individual fatty acid emulsions of EPA, DHA or ALA were equally effective in preventing ventricular fibrillation (Billman et al. 1999). In comparison, an emulsion rich in omega-6 PUFA linoleic acid had no antiarrhythmic effect (Billman et al. 1994; Billman et al. 1997, 1999). However, the arrhythmia prevention was accompanied by an acute fall in heart rate of greater than 20% together with a marked prolongation of the ECG QRS complex, P-R interval and outright 2nd degree heart block in half of the dogs treated with a fish oil emulsion (Billman et al. 1994). These findings demonstrate significant electrophysiological perturbations to the heart that perhaps underpin a powerful acute antiarrhythmic effect of omega-3 fatty acids, but probably do not explain the physiological basis for arrhythmia prevention and reduced cardiovascular mortality with regular fish consumption. For one thing, the lack of selectivity for different omega-3 PUFA contrasts to dietary studies that show purified DHA or EPA+DHA but not EPA alone are

antiarrhythmic at low dietary intakes (McLennan et al. 1996) and whereas dietary ALA exhibits some antiarrhythmic efficacy at high intakes of canola oil, it is not effective when delivered as soybean oil, demonstrating that incorporation of DHA, not circulating ALA is required (McLennan and Dallimore 1995). Meta-analysis shows that, unlike omega-3 LC-PUFA from fish oils, ALA has no significant effect overall on ischaemia or reperfusion induced arrhythmias in animal studies (Matthan et al. 2005) or in the human diet (Mozaffarian 2005) .

The acute infusion findings using the post-myocardial infarction dog model, recently translated into dietary studies, which not only did not replicate the antiarrhythmic effect of acute fish oil or omega-3 PUFA infusion, but rather perversely revealed a pro-arrhythmic effect of a high dietary intake (3360 $mg.d^{-1}$ EPA+DHA) (Billman et al. 2010). While the report acknowledged that the dietary intake responsible for the increased risk of ventricular fibrillation was well beyond human dietary intakes (20 kg dog given equivalent of 13,440 $mg.d^{-1}$ EPA+DHA for an 80 kg man), maximum incorporation of DHA into myocardium was achieved already with the (840 $mg.d^{-1}$) lowest of three dietary doses and further increases in membrane omega-3 LC-PUFA were primarily attributable to an exponential rise in EPA incorporation (Billman et al. 2010). The incorporation of DHA into the myocardial membranes of the dogs is very low compared to other animals and low even compared to human, with or without dietary supplementation (Rocquelin et al. 1985; Sexton et al. 1995; Garg et al. 2006; Metcalf et al. 2007; Metcalf et al. 2010). One human exception is found in the composition of biopsies from heart transplant patients during fish oil supplementation, which demonstrated much lower incorporation of omega LC-PUFA than expected, based on other human samples, more akin to buccal epithelial (cheek) cells and less than in red blood cell membranes of the same patients (Harris et al. 2004). By virtue of the sampling technique that obtained a very small biopsy of the interventricular septum for the purpose of checking

for tissue rejection, the sample may be more representative of endothelial cells than the excitable myocardium. Therefore, it would appear that the dog, whilst a good model of myocardial infarction and ischaemic arrhythmias, is a poor model for investigating the physiological effects of dietary omega-3 LC-PUFA incorporation into myocardium.

4.2. Effects of omega-3 fatty acids on cardiac and cellular electrical excitability

The acute administration of purified fatty acids has a wide range of electrophysiological effects in isolated cell systems and an equally wide range of interactions with arrhythmic stimuli and modulators of cellular electrophysiology that could contribute to their effects on arrhythmia vulnerability, spontaneous beat rate, contractility and relaxation. These ion channels are involved in the genesis and/or maintenance of cardiac action potentials and controlling intracellular stores of Ca^{2+}. They include: Na^{+} currents (I_{Na+}), K^{+} currents (delayed rectifier I_{K+}), transient outward current (I_{TO})) (Judé et al. 2003), and Ca^{2+} current (I_{Ca2+}) (Table 1).

[Table 1 near here]

Isolated myocytes are commonly used for electrophysiological research and have been used extensively to investigate mechanisms of antiarrhythmic effects of omega-3 LC-PUFA. Many observations using isolated cardiomyocytes appear to replicate the acute depressant effects that omega-3 PUFA have on cardiac excitability and conduction after infusion into dogs *in vivo* (Billman et al. 1994). Isolated myocardial cells acutely exposed to omega-3 LC-PUFA become electrically less excitable. Effects such as extreme reduction in the spontaneous beat rate and induction of a slow and sporadic spontaneous contraction pattern (Kang and Leaf 1994, 1995, 1996c, b) are commonly reported (Table 2). When exposed to omega-3 LC-PUFA, these cells require higher stimulation voltages to capture and drive them at a faster beat rate, and exhibit a much lower maximum following-frequency than control cells (Kang and Leaf 1996c). Moreover and perhaps most telling, these acute

electrophysiological effects are shared by all PUFA, including omega-6 PUFA linoleic acid and arachidonic acid. Thus there is little apparent selectivity for the purified omega-3 LC-PUFA (Kang and Leaf 1994, 1995; Kang et al. 1995; Kang and Leaf 1996c) (Table 2.).

[Table 2. near here]

In contrast, cardiomyocytes incubated with EPA or DHA for at least three days exhibit little change in basal spontaneous contractions, yet become protected from the intracellular Ca^{2+} overload and tachyarrhythmias induced by incubation with the Na^{+} pump inhibitor ouabain or the Ca^{2+} channel agonist BayK8644 (Hallaq et al. 1990; Hallaq et al. 1992). Chronic incubation of myocytes with omega-3 LC-PUFA therefore produces effects akin to dietary intervention, with no evidence of the depressed electrical excitability that occurs with acute administration of omega-3 LC-PUFA to cellular incubates or by acute infusion into the dog *in vivo*. Programmed stimulation of ventricular fibrillation in the marmoset heart *in vivo*, is inhibited after chronic fish oil feeding (inducible fibrillation threshold current is higher), but the stimulation voltage required to capture and drive the ventricles above their spontaneous beat rate (electrical excitability) is not affected (McLennan et al. 1993). Nor is capture voltage (electrical excitability) in isolated rat hearts influenced by a fish oil diet, although the current needed to trigger inducible ventricular fibrillation in programmed stimulation is raised significantly (Pepe and McLennan 1996). Consequently, the chronic incorporation of omega-3 LC-PUFA into myocardial membranes appears to inhibit triggered arrhythmias without depressing basal excitability or electrical conduction.

4.2.1. Effects of omega-3 fatty acids on myocardial Na^{+} channel function, K^{+} channel function and arrhythmia

A variety of ion channels contribute to the mammalian action potential, and their modulation has the potential to modify heart rate (spontaneous activation rate) of autorhythmic "pacemaker" cells or arrhythmogenesis in non-autorhythmic cells (Rosen 1991; Lakatta et al.

2010; Rosen and Janse 2010). These channels control ionic currents that create, sustain and curtail cardiac action potentials by the sequential opening and closing of: fast voltage-dependent Na+ channels to initiate an action potential; an outwardly directed K^+ current (transient outward current (I_{TO})) to stabilise the positive membrane potential; a temporarily sustained inward I_{Ca2+} to sustain the action potential plateau; and a second outward current (delayed rectifier) I_{K+}, which moves K^+ out of the cell, repolarising the myocyte to its resting membrane potential (Table 1).

Cellular electrophysiological studies reveal that EPA and DHA acutely inhibit the ventricular sodium current (I_{Na+}). They reduce the current amplitude in neonatal myocardial cells (Xiao et al. 1995), markedly reduce the inactivation voltage and more rapidly restore Na^+ channel inactivation in neonatal or adult myocytes (Xiao et al. 1995; Leifert et al. 1999; Xiao et al. 2004) increase the activation voltage. They also inhibit Na^+ channel ligand binding (Kang and Leaf 1996a). It is postulated that a persistent I_{Na+} could underpin ischaemic arrhythmias, and omega-3 LC-PUFA powerfully block Na^+ channels in non-cardiac cells transfected with a mutant, inactivation-deficient, human myocardial Na^+ channel (Xiao et al. 2006). However, just as chronic incorporation of omega-3 LC-PUFA does not reduce myocyte electrical excitability, the Na^+ channel specific binding is not inhibited after 3 days of cellular incubation (Kang et al. 1997), and three weeks of fish oil feeding prior to myocyte isolation does not increase membrane I_{Na+} activation voltage and has only a minor effect on inactivation voltage (Leifert et al. 2000b) (Table 1). Therefore, both prolonged cellular incubation with omega-3 LC-PUFA and fish oil pre-feeding retain their antiarrhythmic effects (Table 1) in isolated myocytes, independently of effects on Na^+ channels, (Kang et al. 1997; Leifert et al. 2000b) (Table 2).

Acute presentation of EPA or DHA to adult isolated cardiomyocytes alters membrane fluidity and the altered I_{Na+} properties are mimicked by the membrane fluidiser benzyl alcohol (i.e. Na^+ channel blocking activity of the acutely added omega-3 LC-PUFA correlates with changes in membrane fluidity) (Leifert et al. 1999; Jahangiri et al. 2000; Leifert et al. 2000a). However, adult cardiomyocytes isolated from fish oil fed (arrhythmia protected) rats do not display any measurable increase in membrane fluidity (Leifert et al. 2001) and yet retain their antiarrhythmic protection *in vitro*. There may however be some differences between neonatal and adult myocytes because enrichment of membrane phospholipids by 3 days of incubation with EPA did produce an increase in membrane fluidity in neonatal cells (deJonge et al. 1996a). Nevertheless, the balance of evidence suggests that neither the Na^+ channel nor membrane fluidity effects of acutely administered omega-3 LC-PUFA are sufficient to explain the *in vitro* or *in vivo* antiarrhythmic effects of the fatty acids incorporated into cell membrane phospholipid.

Further evidence that the electrophysiological changes in Na^+ channels and electrical excitability may be non-specific comes from the common observation that the omega-6 PUFA linoleic acid, arachidonic acid and the non-metabolisable analogue eicosatetraynoic acids (ETYA) are often equally effective with omega-3 LC-PUFA, and their effects are mimicked by benzyl alcohol (Honore et al. 1994; Xiao et al. 1995; Kang and Leaf 1996a; Xiao et al. 1998; Leifert et al. 1999; Xiao et al. 2006). Antiarrhythmic effects of omega-6 PUFA relative to dietary saturated fat in coronary artery occlusion animal models are long established (Lepran et al. 1981; McLennan et al. 1985, 1988, 1989a; McLennan 1993), and dietary omega-6 PUFA intake has been associated with a downturn in sudden cardiac death in Australia and the USA in the late part of the 20th century (Hetzel et al. 1989). However, in the absence of fish oil fatty acids in the diet the omega-6 PUFA are the primary fatty acids in myocardial cells in hearts vulnerable to arrhythmic stimuli, and when omega-6 PUFA rich

vegetable oils are matched against dietary fish oil directly, the omega-3 LC-PUFA provide a demonstrable antiarrhythmic advantage (McLennan et al. 1988; McLennan et al. 1993). Dietary studies collectively demonstrate both a general antiarrhythmic affect attributable to the relative proportions of PUFA and saturated fat in the diet, and unequivocally that a more potent antiarrhythmic effect of fish oil persists independently of a non-specific PUFA effect (McLennan and Abeywardena 2005) (Figure 3). It is therefore unlikely that effects shared by linoleic acid and DHA or EPA can explain the specific antiarrhythmic or other electrophysiological effects of the omega-3 LC-PUFA.

Only a few studies have focussed on K^+ currents as a target for omega-3 PUFA action. The inhibition of I_{TO} by DHA was demonstrated in transfected hamster ovary cells (Singleton et al. 1999), and has not been evaluated under dietary conditions with chronic membrane incorporation. The delayed rectifier I_{K+} has also only been evaluated in transfected cell systems (Honore et al. 1994; Guizy et al. 2008), and whilst its amplitude is reduced and inactivation enhanced by EPA and DHA, these affects are equally demonstrated for the omega-6 LC PUFA arachidonic acid (Table 1). The study of cardiac ion channels transfected into other cell types may be particularly problematic when considering factors influenced by membrane fatty acid composition, since the membrane composition of different cell types has a marked influence on activity of membrane proteins transposed into them, first noted 30 years ago (Abeywardena and Charnock 1983). The one report on I_{K+} function in adult cardiomyocytes after fish oil feeding found no significant change in activation voltage despite chronic membrane compositional change and reduced arrhythmia vulnerability in the same celluar system (Leifert et al. 2000b). Moreover, it was specifically noted that some of these acute effects on K^+ channels can only be observed when the omega-3 LC-PUFA is applied to the extracellular side of the membrane (Guizy et al. 2008) and therefore is unlikely to play a role in the action of dietary-derived, membrane incorporated fatty acids. Fatty acids that are

then liberated as intracellular signalling molecules after intracellular phospholipase activation (Nair et al. 1997; Siddiqui et al. 2008; Jenkins et al. 2009; Richardson et al. 2011) or influence the activity of intracellular signalling proteins such as protein kinase C (Stillwell et al. 2005).

Many studies using isolated cell systems, including these experiments on I_{K+}, focus on an individual fatty acid (Singleton et al. 1999; Guizy et al. 2008) without controls to detect selectivity of or specificity for omega-3 effects. There is a prevalence of studies evaluating EPA and large gaps in the literature with no data for the principal myocardial omega-3 LC-PUFA, DHA (Guizy et al. 2008). The failure to demonstrate specificity prejudices their translation to interpretation of physiological dietary effects of omega-3 LC-PUFA with any certainty.

The ability of acutely administered omega-3 LC-PUFA to profoundly suppress isolated myocyte spontaneous beat rate (Kang and Leaf 1994) or inhibit I_{Na+} activation (Xiao et al. 2004) is mimicked by the class I antiarrhythmic agent and local anaesthetic lignocaine. Delayed conduction, as produced by acute EPA infusion in dogs (Billman et al. 1994), is also a characteristic property of lignocaine (Singh et al. 1992). Importantly, while acute administration of omega-3 fatty acids may share these properties with the class I antiarrhythmic drugs, there is a contrast in their clinical effectiveness, with the fish oil fatty acids reducing the risk of post-infarction sudden death (Valagussa et al. 1999; Marchioli et al. 2002) but the class I antiarrhythmics increasing the risk through their pro-arrhythmic effects (Singh et al. 1992). The contrasting outcomes of a fish or fish oil intervention trial reducing mortality (Burr et al. 1989) while a major class I antiarrhythmic trial (cardiac arrhythmia suppression trial) of the same vintage was stopped because of excessive arrhythmic mortality (Investigators 1989). may be instructional. Both were conducted in equivalent post-

myocardial infarction patients at increased risk of sudden cardiac death. One caveat is that lignocaine (class Ia) is less potent and may not share all properties of the withdrawn class Ic drugs of that trial (Williams 1991). The reported pro-arrhythmic action of dietary omega-3 fatty acids in dogs (Billman et al. 2012) has not been observed in other experimental studies or clinical epidemiology but implies potential adverse effects of extremely high intakes, producing high membrane incorporation of EPA over DHA (Billman et al. 2010), and this may be related to the non-specific block of Na^+ channel activity.

4.2.2. Effects of omega-3 fatty acids on myocardial Ca^{2+} handling, arrhythmia and myocardial function.

Myocardial I_{Ca2+} not only sustain the action potential plateau, but also provide a window to the cell that can lead to intracellular Ca^{2+} overload, which results from the complex interplay between Ca^{2+} channels, Na^+/Ca^{2+} exchange, sarcoplasmic reticulum Ca^{2+} uptake, release and spillover, and mitochondrial Ca^{2+} uptake. Cardiac arrhythmias can be initiated by a variety of stimuli that have in common the perturbation of cellular Ca^{2+} content or handling. Arrhythmias generated in isolated myocytes by the Na^+ pump inhibitor ouabain, catecholamines, elevated extracellular $[Ca^{2+}]$, anoxia, KCl, the Ca^{2+} ionophore A23187, lysophospholipids, Ca^{2+} channel agonist BayK8644, and hydrogen peroxide, are all associated with elevated intracellular Ca^{2+} $[Ca^{2+}]_i$. The cellular arrhythmic activity and the rise in $[Ca^{2+}]_i$ are both prevented by acute application of omega-3 LC-PUFA (Kang and Leaf 1994, 1996c; Xiao et al. 1997; Rodrigo et al. 1999; Rinaldi et al. 2002) (Table 2). Similarly, Ca^{2+} overload and cellular Ca^{2+} transients are inhibited in neonatal cardiomyocytes after 3-5 days chronic omega-3 LC-PUFA incubation or in adult cardiomyocytes with elevated membrane DHA from fish oil feeding (Hallaq et al. 1990; Hallaq et al. 1992; Leifert et al. 2001; Jahangiri et al. 2006). The omega-3 LC-PUFA inhibit both L- and T-type Ca^{2+} channels (Pepe et al. 1994; Xiao et al. 1997; Macleod et al. 1998; Rodrigo et al. 1999;

Negretti et al. 2000; Danthi et al. 2005; Verkerk et al. 2009b). Spontaneous intracellular "Ca^{2+} sparks" are reduced in amplitude following either omega-3 LC-PUFA acute application (Xiao et al. 1997; Negretti et al. 2000; O'Neill et al. 2002; Honen et al. 2003) or after fish oil pre-feeding (Honen et al. 2003). The Ca^{2+} sparks originate from the spontaneous release of Ca^{2+} from the sarcoplasmic reticulum with the potential to locally raise the sarcolemmal membrane potential to threshold for action potential generation and arrhythmia (Lakatta et al. 2010; Yaniv et al. 2012; Monfredi et al. 2013). Fish oil prevents the asynchronous contractions and the rise in diastolic $[Ca^{2+}]i$ in electrically driven adult cardiomyocytes during hydrogen peroxide superfusion (Jahangiri et al. 2006). Triggered arrhythmias and delayed after-depolarisations induced by rapid pacing of pig cardiomyocytes in the presence of noradrenaline are also associated with greater accumulation of sarcoplasmic reticulum Ca^{2+} content, increased Na^{+}/Ca^{2+} exchange current, and increased L-type I_{Ca2+}, all of which are inhibited in myocytes derived from pigs fed a fish oil diet (Berecki et al. 2007). In contrast, dogs subjected to dietary fish oil but exhibiting no antiarrhythmic effect, also show no change in Ca^{2+} channel activity (Billman et al. 2010), in line with its poor incorporation of DHA.

The minimum electrical current required to trigger ventricular fibrillation (ventricular fibrillation threshold), is critically dependent on $[Ca^{2+}]_i$ (Zaugg et al. 1996) and is elevated in fish oil fed marmoset monkeys (McLennan et al. 1993). In further support of an alteration in cellular Ca^{2+} handling, arrhythmias provoked by ischaemia and reperfusion in the rat isolated working heart were abolished by either fish oil feeding (Pepe and McLennan 1996) or the sarcoplasmic reticulum Ca^{2+} release channel blocker ryanodine but not by ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake (Pepe and McLennan 2002), whereas myocardial oxygen consumption was reduced by either fish oil feeding or ruthenium red, but not by ryanodine (Pepe and McLennan 2002). Ventricular myocytes from fish oil fed rats also have

lower mitochondrial [Ca^{2+}] (Pepe et al. 1999). There is a strong relationship between Ca^{2+} mechanisms in fatal arrhythmias underpinning acute ischaemic episodes in animals and in man (Pepe and McLennan 2002; Clusin 2003) and there appears to be a congruence between the antiarrhythmic actions of dietary omega-3 LC-PUFA *in vivo* and *in vitro* and effects on intracellular Ca^{2+} handling (Table 2). This stands in contrast to the lack of correspondence between the acute and chronic effects of omega-3 LC-PUFA on Na^{+} channels (Table 1) or membrane fluidity in isolated myocytes (Table 2). In contrast, many cellular Ca^{2+}-related mechanisms are modulated by omega-3 LC-PUFA (Siddiqui et al. 2008) and accordingly, the one area of consistency between the acute and chronic (membrane incorporation) effects of omega-3 LC-PUFA relates to cellular Ca^{2+} handling.

4.3. INTRACELLULAR MESSENGERS

4.3.1. Phosphoinositides

Intracellular Ca^{2+} overload underpins cardiac arrhythmias of many different aetiologies, including those stimulated by myocardial ischaemia and reperfusion, digitalis glycosides and catecholamines (Clusin 2003). Fish oil fatty acids inhibit arrhythmias stimulated by all of those conditions. In addition to directly measured effects on Ca^{2+} handling described above, the dietary incorporation of omega-3 fatty acids into myocardial membranes reduces the release of inositol 1,4,5-trisphosphate (IP_3) from (pig) cardiomyocytes in response to alpha-adrenoceptor stimulation (Nair et al. 2000) and from rat hearts in response to ischaemia and reperfusion, simultaneously preventing the corresponding ventricular fibrillation (Anderson et al. 1996). Depletion of intracellular Ca^{2+} stores inhibits both IP_3 release and arrhythmias in isolated hearts or atria subjected to regional ischaemia or simulated ischaemia (Woodcock et al. 1996). Conversely, Ca^{2+} overload stimulates IP_3 release (Woodcock et al. 1996). IP_3, regarded as a Ca^{2+} releasing second messenger, is derived from the cell membrane by the action of phospholipase C on phosphatidyl inositol. Receptor mediated phospholipase C

activity is inhibited in rat cardiomyocytes following incorporation of EPA into membrane phospholipid (DHA not tested) (deJonge et al. 1996a; deJonge et al. 1996b), which could occur as a consequence of altered phosphatidyl inositol fatty acid composition (Charnock et al. 1986) reducing its vulnerability to phospholipase action. Thus, the omega-3 fatty acids appear to interact with cellular Ca^{2+} homeostasis at several levels, potentially allowing them to short-circuit an arrhythmogenic cycle where Ca^{2+} initiates IP_3 release and IP_3 stimulates Ca^{2+} release.

4.3.2. Eicosanoids and other fatty acid derived bioactives

Eicosanoids are a collective term for a series of bioactive compounds derived from 20 carbon PUFA, primarily omega-6 LC-PUFA arachidonic acid. They include prostaglandins, thromboxanes, prostacyclins and the inflammatory mediator leukotrienes (Siddiqui et al. 2008; Jenkins et al. 2009). Modulation of eicosanoid metabolism is implicated in many actions of fish oil fatty acids because EPA can replace linoleic acid as substrate for cyclooxygenase and lipoxygenase enzymes, often leading to the production of less end product and biologically more benign end product (Lee et al. 1992; Siddiqui et al. 2008). Cyclooxygenase products derived from EPA are less arrhythmogenic in isolated myocytes than those derived from arachidonic acid (Li et al. 1997). Furthermore, fish oil feeding has been found to reduce myocardial eicosanoid production in rat, guinea pig and marmoset monkey, with greater inhibition of the proarrhythmic TXA_2 than of the antiarrhythmic prostacyclin PGI_2 (Abeywardena et al. 1991a, b, c; Abeywardena and Charnock 1995; Oudot et al. 1995; Murphy et al. 1999). This change in eicosanoid balance is demonstrable with high intakes of EPA (>4 $g.d^{-1}$) (Fischer and Weber 1983, 1984) and provides a biologically plausible explanation for the increased bleeding tendency and antithrombotic actions of dietary fish oil (Dyerberg et al. 1978), particularly with the more favourable balance towards EPA in endothelium and platelets (Abeywardena et al. 1987; Lee et al. 1992) compared to

high DHA in myocardium. In the heart, with little EPA incorporation, even the simple replacement of arachidonic acid by DHA might contribute to a less arrhythmogenic eicosanoid profile and contribute to the antiarrhythmic actions of fish oil, as seen in isolated cells during hypoxia (Oudot et al. 1995).

In addition to the long recognised bioactive lipid mediator eicosanoids, the recent identification of an extended range of powerful lipid mediator resolvins and protectins derived directly from EPA and DHA via lypoxygenase and aspirin activated cyclooxygenase pathways (Serhan 2004; Weylandt et al. 2012) opens an array of prospective diet-derived effects on myocardial cell functioning responsive to selective incorporation of DHA. Resolvins are named for their principal role in the resolution of inflammation (Serhan 2004), and with inflammation a key component of ischaemia/ reperfusion, myocardial infarction and heart failure (Luo et al. 2009), there is prospect for identification of a role for these compounds in dietary omega-3 LC-PUFA physiological effects. Early research shows that resolvin E1 (derived from EPA) can protect the heart against reperfusion injury, reducing infarct size (Keyes et al. 2010) as effectively as ischaemic preconditioning or fish oil feeding (Abdukeyum et al. 2008). No data are available regarding prevention of arrhythmias in this coronary occlusion model as arrhythmic animals were excluded from analysis of the infarct size. Nor is there any data published about the DHA derived molecules, except for demonstration of the equal effectiveness of resolvin D1 with resolvin E1 in pain prevention and prevention of renal ischaemic damage.

A recently identified, potent series of bioactive cytochrome P450 epoxy and hydroxy metabolites derived from the omega-6 LC-PUFA arachidonic acid are produced in the heart during ischaemia and reperfusion (Jenkins et al. 2009; Arnold et al. 2010; Konkel and Schunck 2011). The epoxyeicosatrienoic acids (EET) and 20-hydroxyeicosatetraenoic acid

(20-HETE) have a range of often opposing effects in the heart, where EET regulate sarcolemma L-type Ca^{2+} channels and ATP sensitive K^+ (K_{ATP}) channels in mitochondria, myocardial sarcolemma and vascular smooth muscle. The mitochondrial K_{ATP} channels modulate reactive oxygen species generation and intracellular protective signalling. In the sarcolemma, opening K_{ATP} channels shortens the cardiac action potential, reducing intracellular Ca^{2+} overload, and is thought to be a crucial mediator of cardiac preconditioning and post-ischaemic cardiac protection (Baxter and Ferdinandy 2001). Activation of vascular K_{ATP} channel leads to membrane hyperpolarisation, reduces Ca^{2+} influx, and is a key determinant of vascular tone (Lu et al. 2006). The K_{ATP} channels respond to the falling ratio of ATP:ADP in ischaemia. At the same time, ischaemia stimulates phospholipase A_2 activity, which acts to cleave arachidonic acid, EPA or DHA from membrane phospholipid, making it available for metabolism by the cyclooxygenase, lipoxygenase and cytochrome P450 pathways (Jenkins et al. 2009). The omega-6 arachidonic acid derived epoxy EET compounds are cardioprotective in ischaemia and reperfusion, decreasing myocardial infarct size and improving functional recovery. However, they are countered by the hydroxy arachidonic acid derivative 20-HETE, which has opposing and detrimental effects in ischaemic injury. In the rat heart, fish oil feeding replaces a proportion of arachidonic acid with DHA (Slee et al. 2010; Konkel and Schunck 2011) and largely reduces the production of arachidonic acid derived cardiotoxic 20-HETE (Abeywardena and Charnock 1995; Konkel and Schunck 2011), replacing it with a sevenfold increase in DHA derived 22-hydroxydocosahexaenoic acid (22-HDoHE) (Konkel and Schunck 2011). There is little evidence of EPA-derived epoxy or hydroxy metabolites in the heart even after fish oil feeding, in line with its low presence on the membrane phospholipids. The arachidonic acid derived metabolites have a range of opposing effects on inflammation and ischaemic organ and cell injury, and it has been established that substantial production of DHA epoxy and hydroxyl metabolites occurs in the heart after fish oil feeding (Arnold et al. 2010; Konkel and

Schunck 2011) However, aside from some evidence that the (potentially cardioprotective) epoxy derivatives of DHA have up to 1000 times the potency of arachidonic derived EET in activating vascular K_{ATP} channels to promote dilation of coronary microvessels (Lu et al. 2002), their cellular effects are, like the resolvins, largely unexplored. With more than 20 different bioactives derived from EPA and DHA there is a strong prospect for their involvement in omega-3 LC-PUFA action and the K_{ATP} channel with its role in ischaemic protection, and preventing Ca^{2+} overload may be a prime target.

5. MYOCARDIAL ENERGETICS OF ARRHYTHMIA AND HEART FAILURE

In addition to direct interaction with arrhythmic mechanisms affecting cellular electrophysiology, dietary fish oil increases myocardial oxygen efficiency in the isolated heart through reducing oxygen demand without reducing cardiac output or external work. This occurs independently of heart rate and provides the heart with enhanced coronary vasodilator reserve (Pepe and McLennan 2002). Vasodilator reserve is the term given to the reserve capacity to dilate coronary arteries to provide increased flow on demand. It is commonly low in conditions of cardiac hypertrophy and heart failure (McLennan et al. 2012), providing limits to exercise capacity without creating ischaemia. In the early reperfusion of ischaemic myocardium (e.g. saved infarct) it is common for contractile function and cardiac output to be depressed (myocardial stunning). This puts the post-ischaemic heart at risk of high sympathetic stimulation to compensate the declining cardiac output through increased heart rate. Dietary fish oil enhances early post-ischaemic myocardial recovery in animals (Demaison et al. 1994; al Makdessi et al. 1995; Pepe and McLennan 2002, 2007; Abdukeyum et al. 2008). Ischaemia and reperfusion is a powerful stimulus for myocardial Ca^{2+} overload (Piper 2000), leading to: increased diastolic Ca^{2+}, retarding relaxation; and increased sarcoplasmic reticulum Ca^{2+}, promoting Ca^{2+} sparks and arrhythmia. Usually a passive bystander in Ca^{2+} homeostasis, the mitochondria acutely become overloaded as well, resulting

in over-activation of Ca^{2+}- dependent energy consuming enzymatic reactions and generation of reactive oxygen species (Peng and Jou 2010). The oxygen sparing cardioprotective action of fish oil may be indirectly mediated through reduced intra-mitochondrial Ca^{2+} altering mitochondrial function (Pepe et al. 1999). It provides a form of nutritional preconditioning that protects against acute ischaemic insult (Abdukeyum et al. 2008), improves post-infarction survival (Zaloga et al. 2006), and promotes work capacity in the hypertrophied and failing heart (McLennan et al. 2012). Ischaemia produces energy wasting metabolic responses stimulated by mitochondrial Ca^{2+} overload (Demaison et al. 1996; Peng and Jou 2010) but fish oil feeding improves post-ischaemic recovery of mitochondrial energy metabolism (Demaison et al. 1994) and reduces futile cycling of reactions consuming oxygen (Grynberg and Demaison 1996). Inhibition of mitochondrial Ca^{2+}-uptake abolishes the dietary-induced differences in cardiac oxygen consumption (Pepe and McLennan 2002). By reducing myocardial oxygen demand, the omega-3 LC-PUFA increase the reserve coronary perfusion capacity (Pepe and McLennan 2002) and the reserve work capacity of the normal (Abdukeyum et al. 2008) or hypertrophied heart (McLennan et al. 2012), raising the threshold of work that the heart can perform before demand creates ischaemic conditions to stimulate changes in intracellular Ca^{2+} that may be arrhythmogenic or conducive to failure.

6. CONCLUSIONS

The mammalian myocardium has in common with other excitable tissue, a preference to accumulate the omega-3 LC-PUFA, DHA and it avidly incorporates high concentrations when they are provided in the diet. Fish oil feeding studies mainly demonstrate physiological effects of increasing DHA in cardiac membranes. Accordingly, DHA is the active component of fish oil in preventing cardiac arrhythmia, lowering heart rate and preconditioning the heart to resist a variety of insults – all are intrinsic effects dependent upon changing the fatty acid composition of the heart. The lowering of triacylglycerols and

blood pressure to influence plaque formation and ischaemic heart disease are extrinsic to the fatty acid composition of the myocardium. Selective antiarrhythmic effects are demonstrable for DHA over EPA when provided as purified fatty acids in the diet of rats (McLennan et al. 1996), and heart rate in healthy humans is selectively lowered by DHA (Grimsgaard et al. 1998). Taken in conjunction with the observation that EPA and DHA are often equivalent in their cellular actions in isolated myocyte systems (Table 1, Table 2), the selectivity for DHA in dietary interventions likely resides partly in its preferential availability within the cell by virtue of its selective incorporation into excitable cell membranes (McLennan and Abeywardena 2005; Arnold et al. 2010). The appearance of antiarrhythmic effects with dietary fish oil feeding coincides in time with DHA incorporation into cell membranes (McLennan 2001; Owen et al. 2004). Critically, the sometime failure of fish oil supplement clinical trials to replicate epidemiological findings may reflect the all too frequent use of supplements rich in EPA and low in DHA, that stands in contrast to the predominant provision of DHA in populations that regularly consume fish.

Whilst international guidelines for fish oil consumption focus on treatment of heart disease, they also make recommendations for cardiovascular health and disease prevention, most commonly ~500 $mg.d^{-1}$ of EPA+DHA for healthy adults (Vannice and Rasmussen 2014). The recommended dietary intakes represent a compromise between the high doses required for triacylglycerol lowering (and for which there is strong randomised control trial evidence) and the lower intakes associated with cardiovascular health in population studies. With a clear delineation of low and high dose effects attributable to intrinsic and extrinsic cardiac physiological roles of omega-3 LC-PUFA respectively (Figure 2), we can now be more certain about accepting the intakes derived from the population studies to optimise healthy heart performance as they are based on cardiac intrinsic effects of fish oil fatty acids. These studies identify 250 $mg.d^{-1}$ EPA+DHA for maximal slowing the heart rate (Mozaffarian et al.

2005c) and reducing risk of sudden death or heart failure (Mozaffarian and Rimm 2006). Considering 100 g of salmon contains approximately 2000 mg EPA+DHA, then 200 g of salmon per week should be plenty. The major consideration before taking supplements is: do you get enough from your diet? Historically, the western diet has become poor in omega-3 PUFA (Leaf and Weber 1988), which is also revealed by the low median intake of EPA+DHA in the modern Australian diet (estimated at 24-60 $mg.d^{-1}$) (Meyer et al. 2003; Howe et al. 2006), perhaps to the point of deficiency (Flock et al. 2013a) and put further at risk as nutritional intake declines with age. There is little evidence to suggest going beyond the 250 $mg.d^{-1}$ EPA+DHA will provide any physiological benefit for a fit and healthy individual, but they might consider opting for a supplement with more DHA than EPA if they are not eating fish.

Whilst a large body of research has clearly and unequivocally identified acute inhibition of myocardial Na^+ currents as a primary target for omega-3 LC-PUFA antiarrhythmic action and heart rate slowing in isolated cell systems (Leaf et al. 2005; Kang 2012), these effects are shared indiscriminately by other PUFA as well as local anaesthetics and alcohol. In other words, they may be attributable to a non-specific detergent-like or membrane stabilisation that is not representative of dietary mediated membrane DHA incorporation. On the other hand, the omega-3 LC-PUFA have cellular electrophysiological effects on Ca^{2+} handling that demonstrate resistance to stimuli that induce cellular Ca^{2+} overload and uncoordinated cytosolic Ca^{2+} fluctuations (Leaf et al. 2005; Kang 2012). The changes in Ca^{2+} handling are not shared by the omega-6 fatty acids *in vitro* and are equally demonstrable in cells harvested from fish oil fed animals. Modulation of Ca^{2+} handling can in turn, directly or indirectly underpin: arrhythmia prevention; heart rate slowing; improved contractile function and relaxation; decreased myocardial oxygen consumption; and resistance to ischaemic injury.

Much of the clinical and epidemiological and animal dietary evidence converges to strongly support a role for arrhythmia prevention and heart rate lowering by fish oil omega-3 fatty acids in reducing heart disease mortality risk. The antiarrhythmic effects and other direct effects on heart function occur at lower doses and are independent of and additional to any beneficial effect on the classical risk factors of plasma triacylglycerols and hypertension. Incorporation of DHA into myocardial membranes is demonstrable with very low supplemental intakes and evidence suggests the effects of consuming fish represent correction of a dietary deficiency rather than a therapeutic effect, supporting a move towards establishing a recommended daily intake for DHA (Flock et al. 2013a). While the range of electrophysiological effects coupled with influences on intracellular second messengers and lower myocardial oxygen demand indicate several potential mechanisms of action and it is likely that no single mechanism is unique in providing antiarrhythmic protection, cellular Ca^{2+} homeostasis appears to be the key to a number of the potential mechanisms. These include electrophysiological and metabolic effects that can be demonstrated both in isolated cells and in the whole heart and could link to second messenger effects and intracellular signalling (Siddiqui et al. 2000; Jenkins et al. 2009). The evidence suggests that antiarrhythmic protection, heart rate slowing and optimised cardiac contractile function are best achieved through regular consumption of fish oor fish oil to promote incorporation of DHA into membrane lipids, however many of the effects observed *in vitro* await validation in dietary modulated cells known to have omega-3 derived arrhythmia protection *in vivo*.

This review identifies the pleiotropic physiological effects of fish oil as either intrinsic – direct effects on heart function dependent upon membrane incorporation, or extrinsic – indirect effects through triacylglycerols, blood pressure and atherosclerosis. Of the significant omega-3 fatty acids, DHA predominates over EPA in fish we eat and is preferentially incorporated into myocardium. Extrinsic effects are achieved only with doses of EPA+DHA

exceeding 3 $g.d^{-1}$, whereas intrinsic effects are derived from usual dietary intakes of less than 1 $g.d^{-1}$, perhaps as little as 250 $mg.d^{-1}$, and include improved efficiency of myocardial oxygen use, contractility, resting heart rate, and nutritional preconditioning of the heart to resist ischaemic injury, heart failure and arrhythmias. DHA is selectively active over EPA in heart rate slowing and arrhythmia prevention. Intrinsic modulation of cellular Ca^{2+} handling provides a biologically plausible physiological basis for both healthy heart function, and nutritional prevention of sudden death, and heart failure. It is worth noting for future investigation, that fish oil nutritional preconditioning of the heart has more than a passing similarity to the sustained cardioprotective effects of exercise training.

The disparate intakes and physiological mechanisms underpinning different clinical endpoints and selectivity for DHA, plus the common employment of high EPA, low DHA oils for clinical trials can perhaps explain the sometimes contradictory outcomes of fish oil clinical trials and provide insight to nutritional optimisation of cardiac function and selection criteria for clinical trials based on an improved hypothesis regarding mechanism of action.

Future basic research, still needed to characterise plausible mechanisms of action of omega-3 LC-PUFA, should concentrate on the fatty acid concentrations and dietary intakes shown to be effective by observational population studies, in order to separate the nutritional from the therapeutic effects. What has been established is that only small additional intkes are required to induce physiological change. The electrophysiological effects observed with acute administration of fatty acids in cellular systems, must be reproducible in cells having dietary derived membrane compositional change. Most importantly, individual fatty acid evaluations should always include DHA, as it is the principal fatty acid incorporated into myocardial membrane phospholipid. The recent discovery of DHA-derived cyclooxygenase, lipoxygenase and cytochrome P450 products promises to be a fruitful area of research. "A

new link between omega-3 fatty acids and cardiac disease" (Westphal et al. 2011) describes the pathways and some effects of the cytochrome P450 epoxy and hydroxy docosanoids derived from DHA. This may explain the many parallels that the physiological effects of dietary fish oil and myocardial membrane DHA share with attenuation of Ca^{2+} overload. Future applied research should focus on experimental or disease models (patient populations) that demonstrate a pathophysiological basis that is relevant to biologically plausible intrinsic mechanisms of action for fish oil fatty acids, avoiding the indiscriminate pooling of populations or outcomes subject to disparate intrinsic and extrinsic mechanisms.

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Figure Legends:

Figure 1.

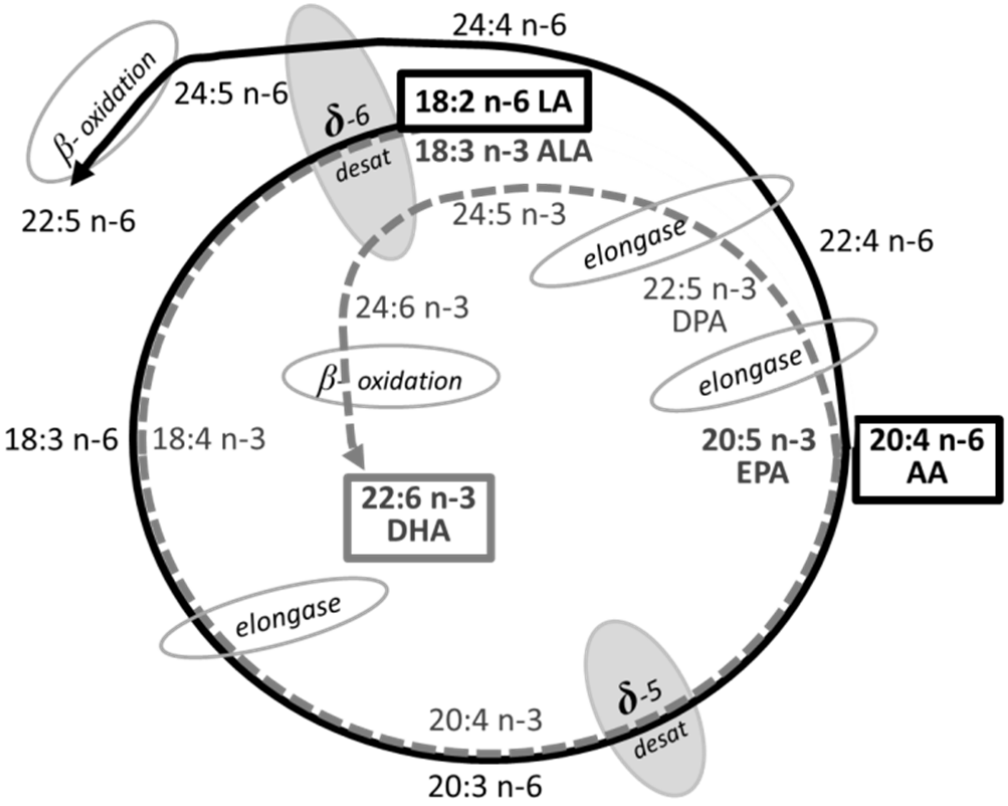
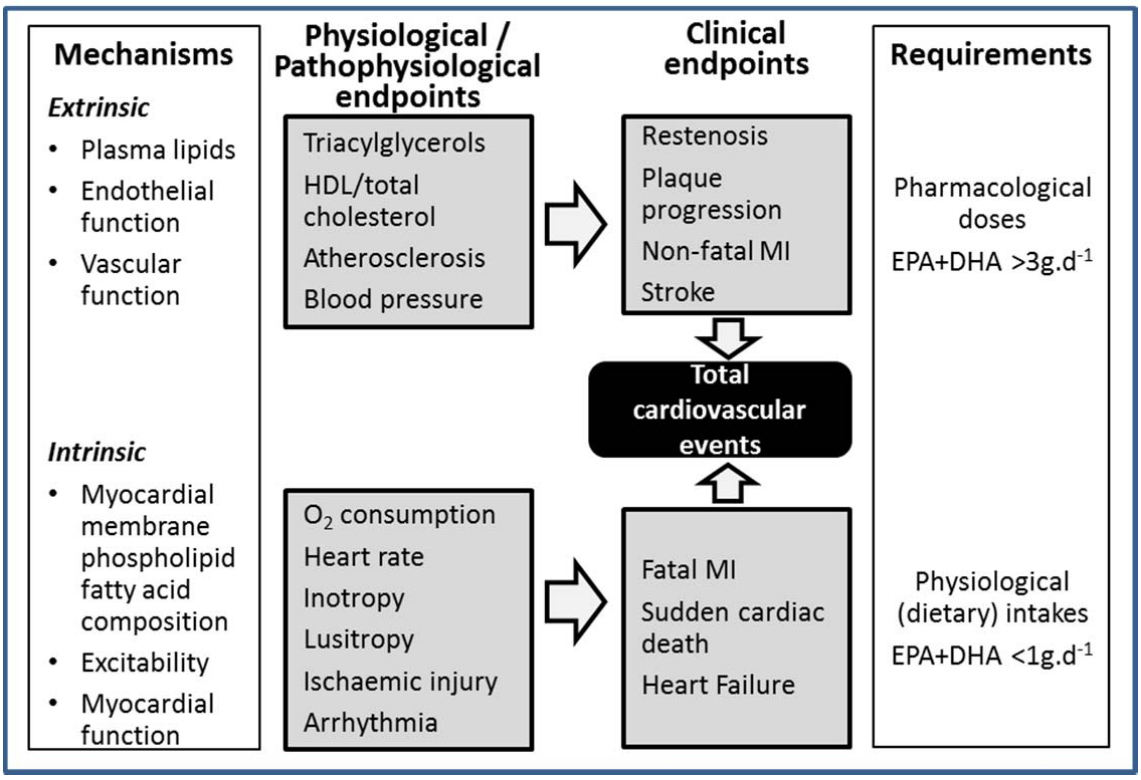


Figure 1.

Schematic pathway of polyunsaturated fatty acids (PUFA) conversion through chain elongation/desaturation, illustrating the main rate-limiting competition point (δ-6 desaturase). Omega-6 PUFA pathway in black with solid line; Omega-3 PUFA pathway in grey with dashed line; Principal dietary sources in **bold**. Principle myocardial membrane PUFA boxed; Fatty acid nomenclature: e.g. 22:6 n-3 - indicates carbon chain length (22): number of double bonds (6) and position of 1st double bond from terminal methyl end of the molecule (n-3).

LA linoleic acid 18:2 n-6; ALA alpha-linolenic acid 18:3 n-3; AA arachidonic acid 20:4 n-6; EPA eicosapentaenoic acid 20:5 n-3; DPA docosapentaenoic acid; DHA docosahexaenoic acid 22:6 n-3; Adapted from: (Gibson et al. 2013)

Figure 2.



Linking omega-3 fatty acid requirements for physiological and clinical endpoints with intrinsic cardiac and extrinsic extra-cardiac mechanisms.

EPA: eicosapentaenoic acid; DHA docosahexaenoic acid

Figure 3.

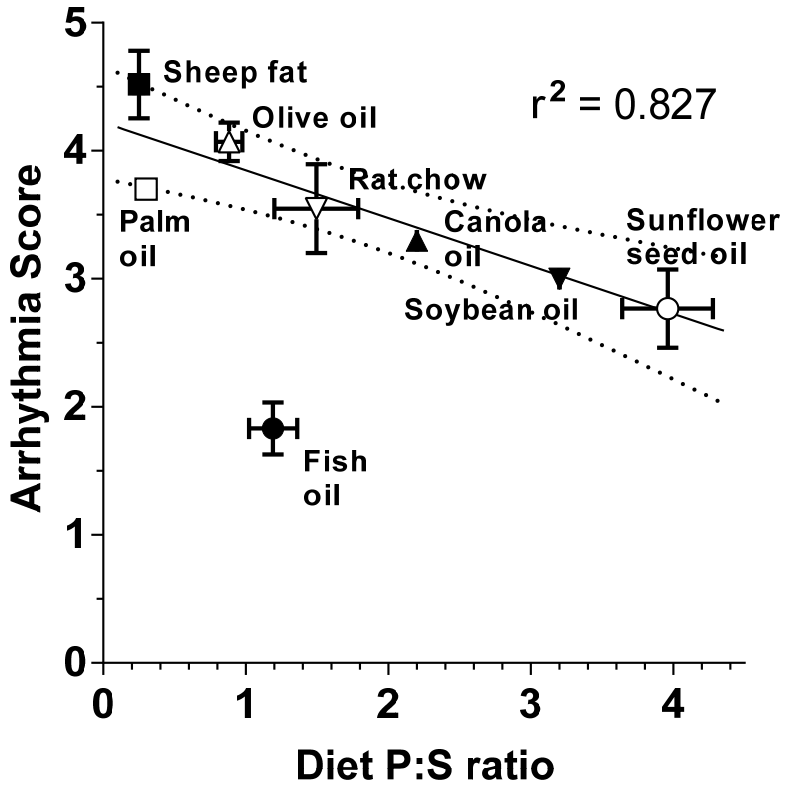


Figure 3. Relationship between the ratio of polyunsaturated to saturated fats (P:S ratio) in rat diets to the arrhythmia score measured during myocardial ischaemia *in vivo* or *in vitro*. ■ sheep fat, □ palm oil, △ olive oil, ▽ reference (rat chow), ▲ canola oil, ▼ soybean oil, ○ sunflower seed oil, ● fish oils. Symbols represent mean P:S ratio for the supplemented diet and mean arrhythmia score. Vertical bars represent SEM for arrhythmia score. Horizontal bars represent SEM for P:S ratio of diets. r^2 correlation coefficient calculated from all data excluding fish oil diets. Solid line represents regression line of best fit, dotted lines represent the 95% confidence intervals. $P < 0.0001$ for slope. Figure adapted from (Grynberg et al. 1997) with data from (McLennan et al. 1985, 1988, 1989a; McLennan et al. 1989b; McLennan et al. 1990; Charnock et al. 1991; McLennan 1993; Siebert et al. 1993; McLennan and Dallimore 1995; Abdukeyum et al. 2008)

Table 1. Summary of influence of fatty acids on isolated cell ionic currents

	I_{Na+}	I_{TO}	Inward rectifier I_{K+}	I_f	L-type I_{Ca2+}
	amplitude ↓, inactivation ↑	inhibited	amplitude ↓, inactivation ↑	↓ density	amplitude ↓
Acute fatty acid presentation					
Selectivity	All omega-3 All omega-6 ETYA, lignocaine	DHA	All omega-3, AA, ETYA	None tested	All omega-3 All omega-6
Specificity	OA, SFA no effect	No other tested	Not effective intracellular		
Chronic fatty acid presentation					
Effective		No diet tested		Fish oil feeding	Fish oil feeding
Not effective	EPA, DHA incubation; Fish oil feeding	No diet tested	Fish oil feeding	High oleic sunflower oil feeding	

↓ decrease; ↑ increase; All acute presentations administered into extracellular incubate; EPA eicosapentaenoic acid 20:5 n-3; DHA docosahexaenoic acid 22:6 n-3; ALA alpha-linolenic acid 18:3 n-3; AA arachidonic acid 20:4 n-6; LA linoleic acid 18:2 n-6; ETYA eicosatetraynoic acid non-metabolisable AA analogue; OA oleic acid 18:1n-9; SFA saturated fatty acid; I_{Na+} Na^+ current; I_{TO} transient outward current; I_{K+} K+ current; I_f funny current; I_{Ca2+} Ca^{2+} current.

Table 2. Summary of influence of fatty acids on isolated myocyte cellular functions

	Spontaneous contraction (neonatal)	**Stimulated Ca^{2+} overload**	**Ca^{2+} sparks**	**Ryanodine receptor**	**Membrane fluidity**
	inhibited, sporadic	inhibited	decreased	↓ binding, ↓ opening probability	increased
Acute fatty acid presentation					
Selectivity	All omega-3 All omega-6 ETYA, lignocaine	EPA, DHA	EPA, DHA	EPA, DHA; Luminal EPA	EPA, DHA, ALA, benzoyl alcohol
Specificity	OA, SFA no effect	AA no effect	No other tested	OA no effect; Luminal - no other tested	LA, OA, SFA no effect
Chronic fatty acid presentation					
Effective	All omega-3 All omega-6 lignocaine	EPA, DHA incubation; Fish oil feeding	Fish oil feeding	None tested	
Not effective	EPA, DHA incubation; Fish oil feeding	AA incubation; Omega-6 sunflower oil feeding	Saturated fat feeding		Fish oil feeding, Saturated fat feeding

↓ decrease; All acute presentations administered into extracellular incubate unless otherwise stated;. EPA eicosapentaenoic acid 20:5 n-3; DHA docosahexaenoic acid 22:6 n-3; ALA alpha-linolenic acid 18:3 n-3; AA arachidonic acid 20:4 n-6; LA linoleic acid 18:2 n-6; ETYA eicosatetraynoic acid non-metabolisable AA analogue; OA oleic acid 18:1n-9; SFA saturated fatty acid;