Effect of omega-3 fatty acids on atrial fibrillation following coronary artery bypass surgery and cardiac calcium handling in humans.

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2011

Palaniappan Saravanan

School of Medicine

Table of Contents

Page

Table of contents……………………………………………………………………...2
List of tables……………………………………………………………….........8
List of figures ………………………………………………………….................11
Glossary…………………………………………………………………………..14
Abstract …………………………………………………………….................18
Declaration…………………………………………………………….................19
Copyright statement……………………………………………………………....20
Contribution statement……………………………………………………………21
Acknowledgements…………………………………………………….................22

Chapter-1: Introduction……………………………………………………..23

1.1: Omega-3 poly unsaturated fatty acids ……………………………….........24
1.1.2: What are omega-3 fatty acids? 25
1.1.3: Omega-3 Vs Omega-6 Fatty acids……………………………….................27
1.1.4: Sources of n-3 PUFA…………………………………………….................28
1.1.5: Dietary intake Vs Supplements of n-3 PUFA……………………….............30
1.1.6: Omega-3 Index as a cardiac risk factor………………………………….......30
1.2: Atrial Fibrillation following cardiac surgery………………………......31
1.2.1 Atrial Fibrillation– a significant health care problem ……………………...31
1.2.2: AF following coronary artery bypass graft (CABG) surgery………………..…....32
1.2.3: Mechanisms of AF following CABG……………………………………...…....32
1.2.4: AF following cardiac surgery as a “model”-Pros & Cons……………………........34
1.3: n-3 PUFA and Cardiac Arrhythmias……………………..........34
1.3.1: n-3 PUFA, Ventricular Arrhythmias & Sudden Cardiac Death………………..…34
1.3.2: n-3 PUFA in Heart Failure and Arrhythmias………………………………….....41
1.3.3: n-3 PUFA and Atrial Arrhythmias…………………………………………..…42

1.4: Effects of n-3 PUFA on Ion Channels, Exchangers and Calcium Handling Proteins……………………………………………………...43

1.4.1: Putative Mechanisms of anti-arrhythmic action of n-3 PUFA …………………...43

1.4.2 Acute administration Vs Incorporated Fatty acids…………………………...........44

1.4.3 n-3 PUFA and cardiac membrane excitability……………………………………...45

1.4.4: n3-PUFA and the Sodium Current ………………………………………………47

1.4.5: n-3 PUFA and the re-polarizing currents ………………………………………...49

1.4.6: n3-PUFA and the Calcium Current ……………………………………………....51

1.4.7: n-3 PUFA and the Sodium-Calcium Exchanger–(NCX)…………………………52

1.4.8: n-3 PUFA and Calcium Regulation ………………………………………………53

1.4.9: n-3 PUFA and expression levels of cardiac ion channel proteins…………………55

1.5: AF and cardiac calcium handling……………………………………...55

1.5.1: Mechanisms involved in cardaic calcium cycling…………………………………56

1.5.2: Diastolic calcium release and pro-arrhythmias…………………………………....57

1.5.3: Alteration in SR function and arrhythmogenic diastolic calcium release in AF…..58

1.5.4: Effect of n-3 PUFA on cardiac calcium regulation………………………………59

1.6: Summary of current evidence…………………………………………59

1.7: Research hypotheses…………………………………………………..60

Chapter-2: Materials and methods……………………………………61

2.1: Clinical methods……………………………………………………62

2.1.1: Study design…………………………………………………………………….62

2.1.2: Sample size calculation…………………………………………………………63

2.1.3: Patient recruitment …………………………………………………….........63

2.1.4: Venous blood sample collection and processing………………………………..65

2.1.5: Echocardiographic assessment…………………………………………………....66

2.1.6: CABG surgery and tissue processing ………...66

2.1.7: Dietary intake of n-3 PUFA……………………………………………………67

2.1.8: Post-operative arrhythmia monitoring…………………………………………67

2.1.9: Therapy, monitoring and follow-up after CABG………………………...........68

2.1.10: Clinical outcome measures………………………………………………....69

2.1.11: Clinical indices influencing risk of arrhythmias……………………………....69

2.1.12: Estimation of serum and tissue levels of n-3 PUFA ……………………….…72

2.1.13: Statistical analysis of clinical data………………………………………….…73

2.2: Quantitative PCR…………………………………………………………74

2.2.1: Total RNA isolation from the right atrial appendage tissue………………………75

2.2.2: Determination of the quantity and quality of the RNA isolated…………………..76

2.2.3: cDNA synthesis- Reverse Transcription…………………………………........76

2.2.4: qPCR with primers specific for the target mRNA……………………………...…77

2.3: Immnuohistochemistry…………………………………………………...80

2.3.1: Antibodies used in Immunohistochemistry……………………………………..81

2.3.2: The immuno-histochemistry protocol……………………………………….…82

2.4: Western blotting……………………………………………………….…82

2.4.1: Protein extraction……………………………………………………………….…84

2.4.2: Protein quantification……………………………………………………………...85

2.4.3: Sample preparation………………………………………………………………..…86

2.4.4: Gel electrophoresis……………………………………………………………...86

2.4.5: Transfer of gel to nitrocellulose membrane……………………………………......88

2.4.6: Blocking the membrane (to prevent non-specific binding)………………………..90

2.4.7: Treatment with primary antibody………………………………………………….…90

2.4.8: Treatment with secondary antibody……………………………………………….91

2.4.9: Detection and quantification of antigen……………………………………………91

2.4.10: Stripping and re-probing with house keeper gene……………………………..92

2.5: Cell isolation from human right atrial appendage……………………..92

2.5.1: Further attempts at human atrial cell isolation…………………………………….96

2.6: Animal experiments-methods…………………………………………97

2.6.1: Ventricular myocyte preparation……………………………………………..…98

2.6.2: Myocyte storage and dye loading……………………………………………...101

2.6.3: Experimental set-up…………………………………………………………....101

2.6.4: Field stimulation……………………………………………………………….102

2.6.5: Measurement of calcium concentration and cellular electrophysiology…………103

2.6.6: Data analysis…………………………………………………………………...104

Chapter 3: Results…………………………………………………...105

3.1: Clinical outcome……………………………………………...106

3.1.1: Demographics and duration of supplementation………………………………106

3.1.2: Drug compliance and levels of n-3 PUFA……………………………………..108

3.1.3: Primary clinical outcome………………………………………………………109

3.1.4: Analyses to assess confounding influence of other variables…………………….112

3.1.5: Analysis of secondary outcomes………………………………………………113

3.1.6: Dietary n-3 PUFA Intake……………………………………………………....115

3.1.7: Postoperative Complications……………………………………………..…116

3.1.8: P-wave parameters in the two groups…………………………………….…116

3.1.9: HRV in the two groups (3rd post operative day)…………………………….…117

3.2: Q-PCR results……………………………………………...118

3.3: Immunohistochemistry-Results……………………………………125

3.4: Western blot- Results………………………………………………132

3.5: Animal Experiments (isolated rat ventricular myocytes)-Results……138

3.5.1: Calcium transient amplitude……………………………………………...138

3.5.2: Rate of decay of systolic calcium transient………………………………139

3.5.3: SR calcium content……………………………………………………….141

3.5.4: Intracellular and sarcolemmal Calcium removal mechanisms…...………………142

Chapter-4: Discussion……………………………………………………143

4.1: Clinical Outcomes……………………………………………………144

4.1.1 N-3 PUFA and AF following CABG……………………………………..144

4.1.2: Summary of clinical studies on the role of n-3 PUFA in AF……………………148

4.1.3: Monitoring for AF episodes in clinical studies…………………………………..152

4.1.4: Conclusion on the effect of n-3 PUFA in AF following CABG…………………153

4.1.5: Is Post operative AF a "Good Model" to study AF?...153

4.1.6: Does n-3 PUFA increase the risk of AF? (Pro-arrhythmic properties of n-3 PUFA)………………………………………………………………………...154

4.1.7: AF, Inflammation and n-3 PUFA as an anti-inflammatory agent………………..155

4.1.8: AF, Autonomic regulation and effect of n-3 PUFA on autonomic function……..156

4.1.9: Effect of n-3 PUFA on expression of m-RNA levels of cardiac autonomic receptors…………………………………………………………………………158

4.1.10: ECG markers of risk of developing AF and the effect of n-3 PUFA on these markers……………………………………………………………………………159

4.1.11: Free and incorporated n-3 PUFA levels and their effect on arrhtyhmias………160

4.2: n-3 PUFA and expression level of ion channels, transporters and calcium handling proteins……………………………………………………..163

4.2.1: qPCR- mRNA levels of cardiac ion channel proteins……………………………165

4.2.2: Effect of n-3 PUFA on expression of m-RNA of cardiac connexins…………….166

4.2.3: Effect of n-3 PUFA on expression levels of calcium handling proteins………....167

4.2.4: Alteration in the expression of cardiac phospholamban…………………………168

4.3: Effect of n-3 PUFA on calcium handling in rat ventricular myocytes.170

4.4: Summary of findings …………………………………………………172

4.5: Study limitations….. …………………………………………………173

4.6: Future directions……………………………………………………...173

4.6.1: Clinical……………………………………………………………………………173

4.6.2: n-3 PUFA, cardiac calcium regulation and AF …………………………………173

References…………………………………………………………...175

Publications and presentations…………………………………………210

Appendices……………………………………………………………211

Word count: 39,584

List of tables

Table 1: Fatty Acid Composition of Human Heart Phospholipids- Data from 53 ventricular biopsy specimens ………………………………………………………….……24

Table 2: Fatty acid composition of the main phospholipid classes in the human heart- Data from 19 biopsy specimens ……………………………………………………….25

Table 3: Omega-3 and Mercury Levels of commonly consumed sea food……………...29

Table 4: Summary of clinical studies on the effect of n-3 PUFA on cardiovascular mortality…………………………………………………………………………..35

Table 5: Summary of animal experiments on the effect of n-3 PUFA on ventricular arrhythmogenesis ……………………………………………………………...39

Table 6: Comparison of concomitant drug therapy and cardiovascular mortality in randomised trials with n-3 PUFA……………………………………………………...41

Table 7: Description of HRV measurements **……………………………………………**72

Table 8: Primer assays for qPCR……………………………………………………...78

Table 9: Primary antibodies used in Immunohistochemistry (±WB)……………………81

Table 10: RIPA buffer and protease inhibitors used during extraction of protein……….85

Table 11: Constituents of Laemmli's sample buffer……………………………….....86

Table 12: Constituents of running buffer…………………………………………………...87

Table 13: Gel concentration and running conditions for proteins of interest……..…… 88

Table 14: Constituents of transfer buffer…………………………………………………...89

Table 15: Constituents of TBS-T…………………………………………………………...90

Table 16: Constituents of transport solution..…………………………………………...**94**

Table 17: Constituents of isolation solution …..………………………………………...95

Table 18: Constituents of normal Tyrode's solution ….…………………………………...95

Table 19: Constituents of isolation solution (2) …………………………………………...97

Table 20: Constituents of Taurine solution……………………………………….......100

Table 21: Baseline characteristics of patients in each treatment group………………...106

Table 22: Serum, PBMC and cardiac tissue levels of n-3 PUFA………………………108

Table 23: Duration of therapy and clinical outcomes…………………………………..110

Table 24: Multivariate analysis to assess influence of confounding variables…………112

Table 25: CRP concentration in the treatment groups………………………………….115

Table 26: Dietary intake of n-3 PUFA………………………………………………….115

Table 27: Effect of n-3 PUFA supplementation on P-wave parameters………………..117

Table 28: Differences in HRV between control and n-3 PUFA group ………………...118

Table 29: Results of qPCR (mRNA) levels of various proteins in human RAA……….120

Table 30: Functional relevance of changes in mRNA levels…………………………...121

Table 31: Semi-quantitative assessment of expression of calcium handling proteins in immuno-histochemistry……………………………………………………………………126

Table 32: Western Blot- Expression of calcium handling proteins……………………132

Table 33: Amplitude of systolic calcium transient……………………………………..139

Table 34: Time taken for the decay of systolic calcium transient from 90% to 50% amplitude……………………………………………………………………………..139

Table 35: Rate of decay of caffeine transient …………………………………………143

Table 36: Summary of studies on the effect of n-3 PUFA on AF (modified from Liu et al with permission) ……………………………………………………………………..150

Table 37: Method of monitoring for AF in studies on the effect of n-3 PUFA on AF (Liu et al with permission) ……………………………………………………………...152

List of figures

Figure 1.1: Schematic diagram of the metabolic pathway of n-3 PUFA

Figure 1.2: Mechanisms involved in cardiac calcium handling

Figure 1.3: Effect of EPA on action potential generation in response to a fixed depolarizing impulse

Figure 1.4: Record of action potential from a guinea pig (left) and rat (right) ventricular myocyte showing control action potential and the effect of addition of 2,5,7.5 and 20 mM EPA (Modified from Macleod JC et al)

Figure 2.1: Clinical trial flow chart

Figure 2.2: Experimental set-up for field stimulation (to measure fluorescence)

Figure 3.1: Kaplan-Meir curve for AF free survival in each group

Figure 3.2: Expression levels of mRNA of calcium handling proteins

Figure 3.3: Expression levels of mRNA of cardiac connexins

Figure 3.4: Expression levels of mRNA of cardiac autonomic receptors

Figure 3.5: Expression levels of mRNA of cardiac sodium channels

Figure 3.6: Expression levels of mRNA of cardiac potassium channels

Figure 3.6 (b): Expression levels of mRNA of cardiac potassium channels (It_o & IK_{ur})

Figure 3.7: Expression levels of mRNA of cardiac calcium channels

Figure 3.8: Immnuofluorescence estimates of calcium handling proteins

Figure 3.9: Immuno-histochemistry: representative images of caveolin expression

Figure 3.10: Immuno-histochemistry: representative images of NCX expression

Figure 3.11: Immuno-histochemistry: representative images of SERCA expression

Figure 3.12: Immuno-histochemistry: representative images of phospholamban expression

Figure 3.13: Immuno-histochemistry: representative images of RYR2 expression

Figure 3.14: Western blot- relative abundance of calcium handling proteins

Figure 3.15: Western blot – expression of NCX- representative image

Figure 3.16: Western blot – expression of SERCA- representative image

Figure 3.17: Western blot – expression of PLBA2- representative image

Figure 3.18: Western blot – expression of RYR2- representative image

Figure 3.19: Rate of decay of systolic calcium transient in n-3 PUFA treated myocytes compared to control myocytes

Figure 3.20: Representative image showing faster rate of decay of systolic calcium transient in n-3 PUFA treated myocytes

Figure 3.21: A typical experimental trace (control cell) illustrating systolic calcium transients followed by application of 10 μM caffeine to release total SR calcium and a gradual return to steady state.

Figure 3.22: Mean amplitude of caffeine transient (F/F_0) in n-3 PUFA Vs control cells

Figure 3.23: Rate of decay of caffeine evoked calcium transient in n-3 PUFA treated cells compared to control myocytes

Figure 4.1: Cumulative effect of n-3 PUFA therapy on incidence of AF following CABG surgery (modified from Liu et al)

Figure 4.2: Effect of n-3 PUFA on the risk of recurrence of AF (modified from Liu et al)

Figure 4.3: A flow diagram of the proposed protective effect of n-3 PUFA during ischemia.

Figure 4.4: Possible mechanisms of action of n-3 PUFA on ion channels

Glossary

α: Alpha

ACEI: Angiotensin converting enzyme inhibitors

AF: Atrial fibrillation

Ag: Silver

$AgCl_2$: Silver chloride

ALA: Alpha linolenic acid

ARB: Angiotensin receptor blockers

β: Beta

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium ion

CABG: Coronary artery by-pass surgery

CaCl2: Calcium chloride

CAV: Caveolin

cDNA: complementary de-oxy ribonucleic acid

CICR: Calcium induced calcium release

COPD: Chronic obstructive pulmonary disease

Ct: Cycle threshold

CX40: Connexin 40

CX43: Connexin 43

DAD: delayed after depolarisations

DHA: Docosa-hexanoic acid

DMSO: Dimethyl sulfoxide

EAD: Early after depolarisations

ECG: Electrocardiography

EGTA: Ethylene glycol tetra-acetic acid

EPA: Eicosa-pentanoic acid

FFA: free fatty acids

HCL: Hydrochloric acid

HDU: High dependency unit

HEPES: Hydroxyethyl-1-piperazineethanesulfonic acid

HRV: Heart rate variability

HRP: Horseradish peroxidase

H_20: Water

ICU: Intensive care unit

IC_{50}: Half maximal inhibitory concentration

$I_{Cal:}$ Calcium current

IgG: Immunoglobulin G

IgM: Immunoglobulin M

I_{Ks}: Slow delayed rectifier current

I_{Kr}: Rapid delayed rectifier current

I_{Kur}: Ultra-rapid delayed rectifier current

$I_{K1:}$ Inward rectifier current

$I_{Na:}$ Sodium current

$I_{NCX:}$ Sodium calcium exchanger current

IQR: Inter quartile range

$I_{to:}$ Transient outward current

I.U.: International Units

KCl: Potassium chloride

Kv4.3: Potassium voltage-gated channel subfamily D, member-3

LA: left atrium

LQT1: Long QT syndrome-1

LV: Left ventricle

$MgCl_2$: Magnesium chloride

µg: Micro-gram

mM: milli-molar

µM: micro-molar

mRNA: Messenger ribonucleic acid

ml^{-1}: per millilitre

n-3: Omega-3

NaCl: Sodium chloride

NaH_2PO_4: Sodium dihydrogen phosphate

NaOH: Sodium hydroxide

NCX: Sodium calcium exchanger

PAGE: Polyacrylamide gel electrophoresis

PBMC: Peripheral blood mononuclear cells

PLB: Phospholamban

PUFA: Poly-unsaturated fatty acids

PVDF: poly-vinylidene fluoride

PWD: P-wave dispersion

qPCR: Quantitative polymerase chain reaction

RAA: Right atrial appendage

RIPA: Radio-Immuno Precipitation Assay

rpm: Revolutions per minute

RT: real time

RYR: Ryanodine receptor

SERCA: Sarco/endoplasmic reticulum calcium adenosine tri-phosphatase

SR: Sarcoplasmic reticulum

SDS: Sodium dodecyl sulphate

TBS: Tris buffered saline

TBS-T: Tris buffered saline with Tween 20

WB: Western blot

Abstract

Omega 3 poly unsaturated fatty acids (n-3 PUFA) have been shown to protect against sudden cardiac death following myocardial infarction and reduce the risk of ventricular arrhythmias in patients with heart failure. At the inception of this study, there was one clinical study that reported n-3 PUFA supplementation reduced the risk of atrial fibrillation (AF) following CABG. As AF is a very common arrhythmia and as there are no safe and effective means of preventing AF, we designed this study to further validate the findings of the previous study in a more robust study design. In addition, this study also aimed to evaluate the cellular changes that underpin the beneficial anti-arrhythmic effect of n-3 PUFA.

The outcome of this study shows that n-3 PUFA does not reduce the risk of AF following CABG. However, short term supplementation with n-3 PUFA reliably increases the membrane incorporation in phospholipids and results in alteration in the expression levels of cardiac calcium handling proteins phospholamban and ryanodine receptors. In addition, such incorporation in animal (rat) ventricular myocytes leads to changes in the rate of decay of the systolic calcium transient and an increase in the amplitude of the caffeine induced calcium transient thereby indicating a greater activity of SERCA. These findings needs further evaluation but is clearly interesting as the clinical situations where n-3 PUFA have been shown to be anti-arrhythmic are situations where cellular calcium overload is the main mechanism of arrhythmogenesis.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

Palaniappan Saravanan
School of Medicine
Faculty of Medical and Human Sciences

Copyright Statement

1) The author of this thesis (including any appendices and/or schedules to this thesis) owns any copyright in it (the "Copyright") and he has given The University of Manchester the right to use such Copyright for any administrative, promotional, educational and/or teaching purposes.

2) Copies of this thesis, either in full or in extracts, may be made only in accordance with the regulations of the John Rylands University Library of Manchester. Details of these regulations may be obtained from the Librarian. This page must form part of any such copies made.

3) The ownership of any patents, designs, trade marks and any and all other intellectual property rights except for the Copyright (the "Intellectual Property Rights") and any reproductions of copyright works, for example graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property Rights and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property Rights and/or Reproductions.

4) Further information on the conditions under which disclosure, publication and exploitation of this thesis, the Copyright and any Intellectual Property Rights and/or Reproductions described in it may take place is available from the Head of School of Medicine.

Contribution statement

1. Inception of the idea and design of the study: Dr. Neil C Davidson, Dr. Stephen C O'Neill and Dr.P.Saravanan.

2. Preparation of protocol, obtaining permission from ethics, obtaining permission from regulatory authorities: Dr.P.Saravanan

3. Recruitment of patients, conduct of the clinical trial, follow-up, clinical data collection and analysis: Dr.P.Saravanan

4. ECG analysis for sub-studies: Dr.P.Saravanan, Mr. Rowan Pollock (clinical scientific officer, Department of Cardiology, Wythenshawe)

5. Molecular biology- QPCR, Western Blot and Immuno-histochemistry: Completely performed by Dr.P.Saravanan with guidance from Dr. H. Dobrzynski and Dr. A. Trafford.

6. Cell isolation from human tissue: Attempted by Dr.P.Saravanan.

7. Animal experiments: Design and methodology by Dr. Stephen O'Neill and Dr.P.Saravanan

8. Planning animal feeds, randomisation and day-to-day conduct of animal experiments: Dr.P.Saravanan

9. Isolation of ventricular myocytes from rats and performing filed stimulation experiments: Dr.P. Saravanan

10. Analysis of data: Dr.P. Saravanan with guidance from Dr. Stephen O'Neill, Dr. H. Dobrzynski and Dr. A. Trafford.

11. Statistical analyses: Dr.P.Saravanan and Ms. Julie Morris, Head of Medical Statistics, UHSM, Wythenshawe, Manchester.

Acknowledgements

I am grateful for the excellent guidance and supportive supervision provided by my supervisor Dr. Stephen O'Neill and my advisor Dr. Cathy Holt.

I am greatly indebted to the relentless support and encouragement provided to me by my clinical supervisor Dr. Neil C Davidson.

My sincere thanks and gratitude to Dr. Halina Dobrzynsky and Dr. Andrew Trafford for their guidance at a very difficult time when I had to change my plans due to difficulties encountered in cell isolation.

I wish to express my thanks to my colleagues Luigi, Kath, Dave, Margaux, Sarah, Louise, Yatong, Mark, Mahesh, Mike and Jessica.

I am thankful to the British Heart Foundation for financially supporting my work with a clinical PhD fellowship.

I wish to express my sincere gratitude to Prof. David Eisner and Prof. Mark Boyett for their support and encouragement.

Last, but by no means the least, this work would not have seen the light of day without the unconditional love that I received from my adorable children- Ilakiya and Dharshan.

Chapter 1

1. INTRODUCTION

1.1: Omega-3 poly unsaturated fatty acids

1.1.1: Fatty acid composition of human cardiac cell membranes

Phospholipids are essential constituents of cell membranes. Changes in content and/or composition of these phospholipids are often associated with alterations in function and may even induce cell damage. Gudbjarnason et al were the first to report that alteration in the composition of fatty acids in membranes of cardiomyocytes was linked to cardio-vascular disease [(1)]. In a study on 53 biopsy specimens of left ventricular myocardium collected during mitral valve replacement surgery, Rocquelin et al [(2)] reported the normal content and composition of various phospholipids in the human ventricular myocardium. Later, the same authors reported that the composition of fatty acids in the membranes of cardiomyocytes from various other parts of the heart such as the papillary muscles and right atrial appendage were similar [(3)] as shown in table-1 & table-2. It is of particular interest to understand the normal composition of fatty acids in various phospholipids seen in human cardiomyocytes, so that a baseline value against which any alteration of content or composition can be compared.

Table-1: Fatty Acid Composition of Human Heart Phospholipids- Data from 53 ventricular biopsy specimens (Rocquelin G et al[2])

Fatty acid methyl ester	Males (29)	Females (24)	Fatty acid methyl ester	Males (29)	Females (24)
14 : 0	0.6 ± 0.04	0.6 ± 0.07	20 : 1 (n − 9)	0.1 ± 0.02	0.1 ± 0.01
15 : 0	0.2 ± 0.02	0.2 ± 0.02	20 : 2 (n − 6)	0.3 ± 0.01	0.3 ± 0.01
16 : 0	15.5 ± 0.38	15.3 ± 0.48	20 : 3 (n − 6)	0.8 ± 0.09	0.7 ± 0.03
16 : 1 (n − 9)	0.5 ± 0.12	0.4 ± 0.04	20 : 4 (n − 6)	23.4 ± 0.45	22.5 ± 0.45
16 : 1 (n − 7)	0.8 ± 0.04	0.6 ± 0.09	20 : 5 (n − 3)	0.5 ± 0.08	0.5 ± 0.03
17 : 0	0.4 ± 0.02	0.4 ± 0.02	22 : 0	0.8 ± 0.04	0.8 ± 0.11
18 : 0	14.4 ± 0.18	14.1 ± 0.15	22 : 1 (n − 9)	Tr.	Tr.
18 : 1 (n − 9)	7.5 ± 0.19	7.6 ± 0.20	22 : 4 (n − 6)	0.5 ± 0.03	0.5 ± 0.05
18 : 1 *trans*[a]	0.7 ± 0.04	0.8 ± 0.05	22 : 5 (n − 6)	0.4 ± 0.02	0.4 ± 0.02
18 : 1 (n − 7)	2.3 ± 0.06	2.2 ± 0.07	22 : 5 (n − 3)	1.6 ± 0.04	1.5 ± 0.06
18 : 2 (n − 6)	18.9 ± 0.42	19.4 ± 0.61	22 : 6 (n − 3)	5.1 ± 0.19	5.3 ± 0.20
18 : 3 (n − 3)	0.2 ± 0.02	0.2 ± 0.02	24 : 0	0.5 ± 0.07	0.6 ± 0.13
20 : 0	0.3 ± 0.02	0.4 ± 0.05	24 : 1 (n − 9)	0.9 ± 0.12	1.1 ± 0.18

Values (%) are expressed as the mean ± S.E.M. Number of subjects are given in parentheses.
[a] Including different positional isomers.
Tr. = traces (less than 0.1%).

Table 2: Fatty acid composition of the main phospholipid classes in the human heart- Data from 19 biopsy specimens (Rocquelin G et al[3])

Fatty acid methyl ester	PC (19)	PE (19)	DPG (19)	SM (19)
16 : 0	33.4 ± 1.84	5.4 ± 0.56	4.4 ± 0.76	23.3 ± 0.03
16 : 1 (n − 9) + (n − 7)	0.8 ± 0.06	0.4 ± 0.04	1.7 ± 0.15	0.5 ± 0.12
17 : 0	0.6 ± 0.06	1.3 ± 0.40	1.0 ± 0.24	1.3 ± 0.21
18 : 0	9.5 ± 0.66	30.1 ± 2.39	5.4 ± 0.71	19.9 ± 1.39
18 : 1 (n − 9)[a]	12.8 ± 0.60	3.0 ± 0.40	6.1 ± 0.48	1.6 ± 0.22
18 : 1 (n − 7)	2.6 ± 0.11	1.4 ± 0.15	6.4 ± 0.44	0.8 ± 0.15
18 : 2 (n − 6)	14.1 ± 0.77	2.5 ± 0.14	67.5 ± 2.46	0.6 ± 0.10
18 : 3 (n − 3)	0.2 ± 0.01	0.8 ± 0.17	0.3 ± 0.06	Tr.
20 : 0	0.2 ± 0.03	Tr.	0.3 ± 0.07	6.2 ± 0.26
20 : 2 (n − 6)	0.3 ± 0.05	0.6 ± 0.06	1.2 ± 0.26	—
20 : 3 (n − 6)	0.8 ± 0.10	0.4 ± 0.05	0.2 ± 0.04	—
20 : 4 (n − 6)	13.0 ± 0.61	30.6 ± 1.44	2.6 ± 0.32	0.6 ± 0.10
20 : 5 (n − 3)	0.2 ± 0.03	0.5 ± 0.06	Tr.	—
22 : 0	Tr.	—	0.2 ± 0.07	19.1 ± 1.27
22 : 1 (n − 9)	—	—	—	0.4 ± 0.08
22 : 4 (n − 6)	0.3 ± 0.05	0.7 ± 0.12	—	—
22 : 5 (n − 6)	Tr.	0.6 ± 0.06	—	—
22 : 5 (n − 3)	0.8 ± 0.07	1.7 ± 0.10	0.9 ± 0.37	—
22 : 6 (n − 3)	1.7 ± 0.11	6.1 ± 0.35	0.2 ± 0.07	—
24 : 0	—	—	—	11.3 ± 0.68
24 : 1	—	—	—	12.2 ± 1.81

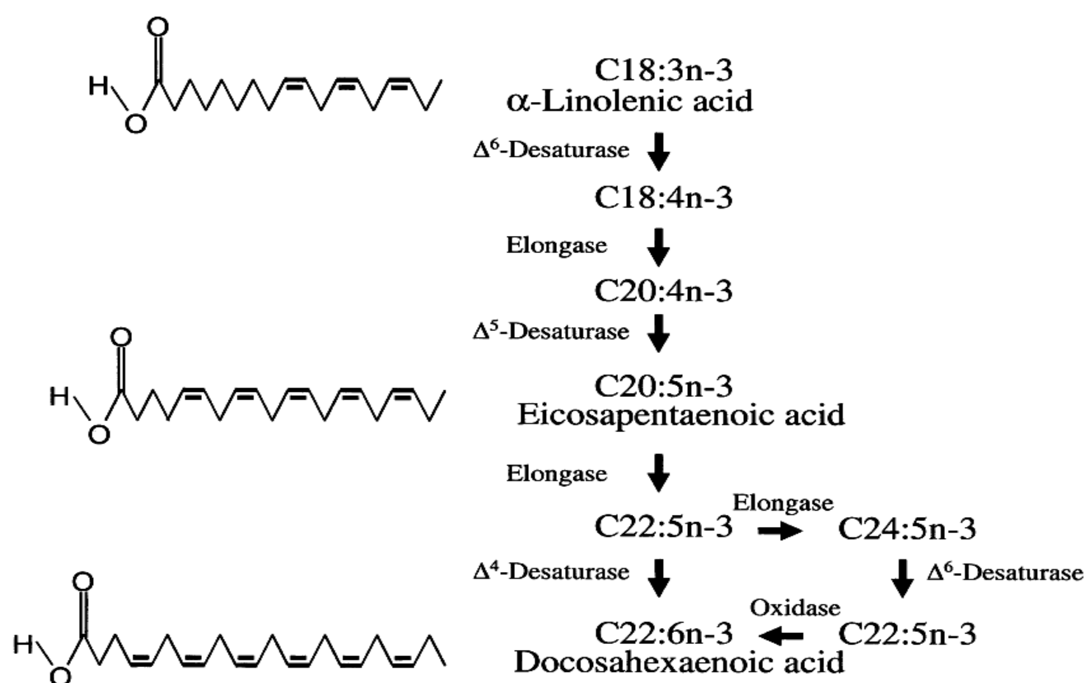
Values (%) are expressed as the mean ± S.E.M. Number of subjects are given in parentheses.
PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; SM, sphingomyelin.

1.1.2: What are omega-3 fatty acids?

The term omega-3 (or n-3) signifies that the first double bond exists as the third carbon-carbon bond from the terminal methyl end (n) of the carbon chain. They are termed poly unsaturated fatty acids as they have multiple double bonds and these are essential fatty acids in humans as humans cannot synthesize them. However, the plant n-3 poly unsaturated fatty acid (n-3 PUFA) α-linolenic acid can be converted to other functionally important n-3 PUFA. The n-3 PUFA that are important in human nutrition are: α-linolenic acid (18:3, n−3; ALA), eicosapentaenoic acid (20:5, n−3; EPA), and docosahexaenoic acid (22:6, n−3; DHA). These three poly-unsaturates have 3, 5 or 6 double bonds in a carbon chain consisting of 18, 20 or 22 carbon atoms, respectively. All double bonds are in the cis-configuration i.e. the two hydrogen atoms are on the same side of the

double bond, where they are more easily transformable. n-3 compounds are fragile and easily subject to transformation because the last double bond is geometrically and electrically more exposed, notably in the natural cis-configuration. The metabolic pathway by which the plant n-3 PUFA, ALA, can be converted to the more functionally relevant n-3 PUFA such as EPA and DHA are shown in figure-1.1.

Figure1.1: Schematic diagram of the metabolic pathway of n-3 poly un-saturated fatty acids



The bio-chemical numbering of the carbon in fatty acids begins from the n (ω) carbon. Note that from the n end, the first double bond appears as the third carbon-carbon bond, hence the name "n-3". This is explained by the fact that the "n" end is almost never changed during physiologic transformations in the human body, as it is more stable energetically.

A recent work by McLennan et al [4] defines the baseline values of the total (in all the phospholipids) n-3 PUFA (EPA and DHA) in the human heart. This study evaluated

the fatty acid composition of myocardial samples from fresh explanted failing and donor hearts available through a heart transplant program. The study revealed that DHA was significantly higher than EPA in all chambers and the n-6 PUFA arachidonic acid (AA) and linoleic acid (LA) were the predominant PUFA. This study also reported that the DHA and EPA content, but no other PUFA, increased with age. Comparison of chambers showed total n-3 PUFA content of human atria were significantly higher than the ventricles while the total n-6 PUFA content was higher in the ventricles than in the atria. Thus, this study concluded that DHA is the principal n-3 PUFA in the human heart and it increases with age.

1.1.3: Omega-3 Vs Omega-6 Fatty acids

There is considerable debate about the importance of the ratio of n3 and n-6 fatty acids in health and disease. The parent omega-6 fatty acid in human diet is linoleic acid (LA) present in abundance in plant oils. LA is then converted to arachidonic acid (AA) which is the precursor for the production of pro-inflammatory cytokines. LA is converted to AA by the action of the enzyme Δ-6 desaturase, which is the same enzyme necessary to de-saturate alpha-linoleic acid (ALA), the parent compound of the omega-3 class. Thus LA and ALA compete for this desaturase. The presence of ALA in the diet can inhibit the conversion of the large amounts of LA in the Western diet, which contains high amounts of plant oils rich in omega-6 PUFAs (e.g. corn, safflower, and soybean oils) [(5)] . Thus the n-6: n-3 ratio will significantly influence the ratio of the ensuing eicosanoids with the n-6 derived compounds (e.g. prostaglandins, leukotrienes, thromboxanes) [(6)] being more inflammatory than those derived from n-3 PUFA. Studies have reported that a healthy ratio of n-6: n-3 ranges from 1:1 to 4:1 [(7)] While a typical western diets provide ratios of between 10:1 and 30:1 with a substantially higher n-6 content [(8)].

1.1.4: Sources of n-3 PUFA

The plant source of n-3 PUFA is α-Linolenic acid (ALA), which is an organic compound found in many common vegetable oils. Oils derived from plant seeds are the richest sources of α-linolenic acid, notably those of rapeseed (canola), soybeans, walnuts, flaxseed (Linseed oil) and hemp. ALA can be converted to EPA and DHA in humans by the above reaction depicted in figure-1.1. However, the most widely available and a rich source of EPA and DHA is oily fish such as salmon, herring, mackerel, anchovies and sardines. Oils from these fish have a profile of around seven times as much n-3 as n-6. Other oily fish such as tuna also contain n-3 in lesser amounts. Although fish is a dietary source of n-3 fatty acids, fish do not synthesize them, they obtain them from the microalgae or plankton in their diet (9).

One of the issues with consuming large amount of sea food as a source of n-3 PUFA is the risk of ingesting toxic substances particularly mercury. This is, however, present in negligible quantities in most commonly consumed salt water fish. The American Heart Association has published a summary of the n-3 PUFA content and the mercurial content of some of the commonly consumed fish (10) as shown in table 3.

Table-3: Omega-3 and Mercury Levels of commonly consumed sea food

	Omega-3 fatty acids (grams/ 3-oz. serving)	Mean mercury level in parts per million (ppm)
Canned tuna (light)	0.17–0.24	0.12
Shrimp	0.29	Negligible
Pollock	0.45	0.06
Salmon (fresh, frozen)	1.1–1.9	0.01
Cod	0.15–0.24	0.11
Catfish	0.22–0.3	0.05
Clams	0.25	Negligible
Flounder or sole	0.48	0.05
Crabs	0.27–0.40	0.06
Scallops	0.18–0.34	0.05

Fishes with the Highest Levels of Mercury (about 1 ppm)

	Omega-3 fatty acids (grams per 3-oz. serving)	Mean mercury level in parts per million (ppm)
Tilefish (golden bass or golden snapper)	0.90	1.45
Shark	0.83	0.99
Swordfish	0.97	0.97
King mackerel	0.36	0.73

1.1.5: Dietary intake Vs Supplements of n-3 PUFA

Dietary intake is the most desirable way to increase n-3 PUFA intake, but in several clinical situations where n-3 PUFA have been shown to be beneficial in clinical studies, the doses used as supplements vary from 1 g per day of purified n-3 PUFA (Omacor™-which contains 880mg of eicosapentaenoic acid and docosahexaenoic acid in a 1.2:1 ratio) to 2.5-4 grams/day. 1capsule of 1g of Omacor contains n-3 PUFA which is equivalent to the fish oil present in about 55–85 g of fresh tuna, sardines, salmon, or trout, and 652 g of cod fish(11). Such high intakes are difficult to achieve in most parts of the world by dietary means alone. This finding is supported by a study by the EUROACTION study group (12). In this study, despite the fact that the active interventional approaches more than doubled the number of subjects consuming recommended levels of oily fish (8% Vs 17%), increase of long-term changes in diet was poor despite household re-education. Hence, an argument could be made for prescribing supplements in all clinical situations where a reliable increase in n-3 PUFA intake is indicated.

1.1.6: Omega-3 Index as a cardiac risk factor

In a study by Harris and von Schacky (13), it was reported that the measure of the content of EPA and DHA in red blood cells, termed the omega-3 index, could be used as an indicator of n-3 PUFA intake, and thus target dietary modification or supplementation to achieve optimum values of this index. An omega-3 index of 8% or higher has been reported to be associated with the greatest cardiac protection, whereas an index of 4% or less gives the least cardiac protection. This approach, if validated in prospective clinical trials, would be a novel and potentially modifiable risk factor for death due to cardiac disease.

1.2: Atrial Fibrillation following cardiac surgery

1.2.1 Atrial Fibrillation– a significant health care problem

Atrial fibrillation (AF) is the most common sustained heart rhythm disturbance, affecting up to 1 in 4 people at some stage of their life[14] and 3-5% of those over the age of 65 years[15,16]. The presence of AF is associated with approximately a doubling of mortality risk after adjustment for co-existent cardiovascular risk factors[17], with the excess mortality attributable to thrombo-embolic stroke [18,19]. A rapid heart rate response to exercise, beat-to-beat irregularity and loss of the atrial contribution to ventricular filling leads to diminished cardiac output and reduced exercise capacity. This condition is also a major drain on health care resources with approximately one third of all hospital admissions due to arrhythmias being directly related to AF[20]. Current drug treatments are often ineffective, frequently causing adverse effects due mainly to their non selectivity of action and the potential risk of inducing more serious ventricular arrhythmias. It is therefore important to improve current understanding of the mechanisms causing this rhythm disturbance and to explore new therapeutic options that would be safer and effective. Fish oils (Eicasopentanoic acid (EPA) and Docasohexanoic acid (DHA)) have been shown to be beneficial in suppressing ventricular arrhythmias and their role in controlling atrial arrhythmias is actively being evaluated. However, their mode of action is unclear. This study aims to assess the clinical effect of fish oil supplementation on the incidence of atrial fibrillation following coronary artery bypass surgery and compare the electrophysiological properties of atrial myocytes obtained from the supplemented sub group of patients with matched controls.

1.2.2: AF following coronary artery bypass graft (CABG) surgery

Atrial arrhythmias occur after cardiac surgery in 10% to 65% of patients[21,22] with variation according to patient profile, type of surgery, method of arrhythmia surveillance, and definition of arrhythmia. A meta-analysis of 24 trials[22] estimated the incidence of atrial fibrillation after coronary artery bypass surgery (CABG) at 26.7%. The highest incidence of atrial fibrillation is seen on postoperative days 2 to 3, with fewer patients developing atrial fibrillation either in the early postoperative period or 4 or more days after surgery [21,23-26]. The development of atrial fibrillation is associated with a longer hospital stay and increased long-term mortality rate after adjustment for known associated risk factors[27]. There are several preventive measures such as pre-operative use of anti-arrhythmic drugs, atrial pacing techniques etc that have been tried in clinical studies and have been shown to be of some benefit, but most of these therapies are of limited value in suppressing this arrhythmia and/or may be associated with significant adverse effects that they have not been absorbed into routine clinical practice. Hence there is a pressing need to identify an agent that would be effective and safe to use in this clinical setting in particular and in the management of all types of atrial fibrillation in general.

1.2.3: Mechanisms of AF following CABG

During atrial fibrillation, multiple re-entrant wavelets of excitation circulate throughout the atria. Recent work shows that these re-entrant wavelets may be triggered and /or driven by rapidly firing foci in the myocardium of the posterior left atrium and around the ostia of the pulmonary veins [28]. Slowed atrial conduction velocities, altered refractory periods and in particular regional variations in these parameters provide the substrate which facilitates the persistence of atrial fibrillation [29,30]. The risk factors for AF after coronary artery surgery appear to mirror those for spontaneous AF in the general population. Post-operative AF is associated with a prior history of the arrhythmia, is more common with advancing age, occurs

more frequently in men than in women and is associated with prolonged P wave duration on the surface ECG (indicating slowed atrial conduction)[31]. Both AF and coronary artery disease are strongly associated with hypertension and male sex and therefore as a group, patients undergoing coronary artery surgery are at relatively high risk of developing this arrhythmia. Potential triggering factors after coronary artery surgery include increased sympathetic tone[32], ischaemia and trauma to the atrial myocardium, either directly as a result of surgical incisions or as a result of cardioplegic solutions[33-36]. However since the incidence of atrial fibrillation is not consistently lower after "off-pump" cardiac surgery (with no cardiopulmonary bypass), which does not usually involve atrial incisions, it seems that atrial trauma may not play a major role as a cause of AF after CABG[37]. The available evidence suggests that AF after CABG occurs as a result of increased triggering activity, probably related to high levels of sympathetic tone, in a group of patients who are already at risk for the arrhythmia. This is supported by the finding that beta-adrenergic blocking drugs can reduce the incidence AF in the post-operative period[38]. In addition, there is increasing evidence to suggest that there is a role for mediators of inflammation in the occurrence of this arrhythmia particularly following cardiac surgery and this has been borne out by evidence of a significant difference in the levels of pro-inflammatory cytokines in the subset of patients who develop this arrhythmia.

n-3 PUFA have been shown in clinical studies to have inhibitory effects on these pro-inflammatory cytokines. In summary, it is clearly evident that post-operative AF is an important clinical issue and there is an urgent need to identify an agent that will be of significant benefit in reducing the incidence of this common post operative arrhythmia while minimizing pro-arrhythmic tendencies. Fish oils may fit this description perfectly if the benefits shown in a small clinical trial[39] could be reproduced in a well designed, randomized, blinded clinical trial.

1.2.4: AF following cardiac surgery as a “model”-Pros & Cons

AF is a common complication following cardiac surgery and has significant morbidity and mortality associated with it. As this arrhythmia has such high incidence and it happens shortly after surgery, it lends itself as an excellent candidate to test the effect of an intervention on this common arrhythmia. In addition, any intervention that might reduce the risk of this arrhythmia is likely to be potentially useful and will have a direct clinical application in the pre-specified cohort of patients. However, using post operative AF as a surrogate to test the effect of an intervention on AF seen in other clinical settings has its own limitations. The main limitation relates to the fact that the cause of AF following CABG is multi-factorial and does not mimic the aetio-pathology of common clinical AF. There is a considerable amount of acute inflammation, direct myocardial injury due to atriotomy scar and use of multiple inotropic drugs which would have a substantial influence on the occurrence of this arrhythmia which is not common with other forms of clinical AF. The major advantage is the fact that atrial tissue (particularly right atrial tissue) is readily available during surgery thereby allowing the possibility of studying the cellular effects of an intervention and correlating it with the clinical outcome.

1.3: n-3 PUFA and Cardiac Arrhythmias

1.3.1: n-3 PUFA, Ventricular Arrhythmias & Sudden Cardiac Death

Several observational and interventional studies report that high intakes of n-3 PUFA reduced risk of cardiovascular mortality and sudden cardiac death, especially in patients with previous myocardial infarction as shown in table-4

Table 4: Summary of clinical studies on the effect of n-3 PUFA on cardiovascular mortality

Study Type	Authors	Publication details	Main Findings
Observational	Bang Ho et al	Advances in Nutrition research-1980	Low rates of coronary heart disease (CHD) death among Greenland Eskimos consuming large amounts of seafood
Observational	Albert CM et al. (US Physicians Health Study).	JAMA-1998	Consumption of fish at least once per week may reduce the risk of sudden cardiac death in men
Epidemiological	Albert CM et al	NEJM-2002	n–3 PUFA was associated with a reduced risk of sudden death among men without evidence of prior cardiovascular disease.
Epidemiological	Lemaitre RN et al. (Cardiovascular Health Study)	AJCN-2003	Higher intake of n-3 PUFA may lower the risk of fatal ischemic heart disease in older adults.
Epidemiological	Streppel MT et al	Eur Heart J-2008	Fatty-fish consumption lowered risk of SCD. There was no clear relationship between dose of n-3 PUFA and risk of SCD.
Clinical trial (Post MI with dietary Intervention)	Burr M et al. (DART study)	Lancet-1989	A modest intake of fatty fish (two or three portions per week) may reduce mortality in men who have recovered from MI.
Clinical trial (Post MI with supplements)	GISSI-Prevenzione investigators	Lancet-1999	Treatment with n-3 PUFA, lowered the risk of overall and cardiovascular death.
Clinical trial (Stable CAD with dietary advice)	Burr M et al. (DART-2 study)	EJCN-2003	Men advised to eat oily fish, and particularly those supplied with fish oil capsules, had a higher risk of cardiac death. Particulalry increased risk of SCD.
Clinical trial (Secondary prevention in Haemodialysis patients, supplements)	Svensson M et al	Clin J Am Soc Nephrol. 2006	n-3 PUFA supplementation did not reduce the total number of cardiovascular events and death in this high-risk population but significantly reduced the number of myocardial infarctions as a secondary outcome
Clinical trial (Primary prevention with supplements)	Yokotama M et al. (JELIS study)	Lancet-2007	No benefit in major coronary events in the primary prevention group, however, in the secondary prevention sub group, reduction in non-fatal coronary events but not cardiovascular mortality.
Clinical Trial (Heart failure with supplements)	GISSI-HF investigators	Lancet-2008	Reduction in all cause mortality but not SCD. However, considerable reduction in death due to presumed arrhythmias.
Meta analysis	Bucher HC et al	Am J Med.-2002	Dietary and non-dietary intake of n-3 PUFA reduces overall mortality, mortality due to MI, and sudden cardiac death in patients with coronary heart disease.
Systematic review	Leon H et al	BMJ-2009	n-3 PUFA supplementation was associated with reduction in deaths from cardiac causes but had no effect on arrhythmias or all cause mortality.
Systematic review	Zhao YT et al	Ann Med.-2009	n-3 PUFA has a beneficial effect on prevention of SCD in patients with prior MI but not in patients who have angina.

The most convincing evidence for a protective role of n-3 PUFA against sudden cardiac death comes from a sub-analysis of the GISSI-Prevenzione study [40] showing a significant reduction (p=0.04) within 4 months after a myocardial infarction. The presumed mechanism of such benefit would be a reduction in life threatening ventricular arrhythmias—the most common cause of sudden cardiac death in the early stages after a myocardial infarction.

The role of n-3 PUFA in reduction of risk of sudden cardiac death in patients with non-ischaemic cardiac disease is unknown and very little investigation has been done in this area. Investigators of a study [41] in a small number of patients with dilated cardiomyopathy reported beneficial alterations in known risk indicators for sudden cardiac death. In the GISSI Heart Failure study,[42] in which half of participants had heart failure attributable to non-ischaemic causes, sudden cardiac death was not greatly reduced. However, the greatest proportion of reduction in the primary endpoints of total mortality and hospital admission was attributed to a reduction in such events because of a presumed arrhythmic cause.

Anti-arrhythmic potential of n-3 PUFA was tested in patients with an automatic implantable cardioverter defibrillator. Results of such studies have reported inconsistent results with one study showing marginal benefit,[43] another no effect,[44] and a third,[45] suggesting a possibility of increased risk of ventricular arrhythmic episodes in patients whose qualifying arrhythmia was ventricular tachycardia rather than ventricular fibrillation. An absence of overall effect was also reported in a meta-analysis[33] of these studies and in a systematic review of studies on mortality and arrhythmias, including a study of an appropriate implantable cardioverter defibrillator therapy as a marker of arrhythmic burden.[46] Moreover, one clinical study[48] reported that patients with coronary artery disease without previous myocardial infarction could have a heightened risk of sudden cardiac death with a high n-3

PUFA intake. Although this study had methodological limitations, evidence from studies in laboratory animals showed that n-3 PUFA in the presence of coronary ischaemia, without previous myocardial infarction, might predispose to an increased risk of ventricular arrhythmias [(49)]. The conflicting finding and the apparent absence of benefit in studies designed to assess a direct anti-arrhythmic effect could be attributable to differences in the mechanisms of arrhythmia initiation in subsets within these study populations.

The two common mechanisms of initiation of life threatening ventricular arrhythmias are triggered activity and re-entry. Of the cellular electrophysiological effects of n-3 PUFA, shortening of action potential duration [(50)] and slowing of impulse conduction,[(50)] which would affect triggered activity with a favourable outcome, could promote re-entry in a susceptible substrate. Thus, a given patient could have either a lowered or raised risk of serious ventricular arrhythmias on the basis of the mechanism of initiation of the arrhythmia [(51)]. Thus patients who have had a recent myocardial infarction and heart failure, with triggered activity as the predominant mechanism of arrhythmia initiation, would have a beneficial reduction in arrhythmias, whereas those with ischaemic heart disease in the absence of previous myocardial infarction and any other clinical situation in which the predominant mechanism of arrhythmia initiation is re-entry could be expected to have heightened arrhythmic risk. This finding is especially important because it suggests that patient selection could be a crucial issue before starting therapy with n-3 PUFA.

In experimental studies, mostly done in laboratory animals, researchers have reported that n-3 PUFA have several potential anti-arrhythmic effects [(52,53)] most notably a direct effect on cardiac ion channels. Initial data [(53)] from single-cell experiments with isolated

cardiomyocytes showed that acute application of purified n-3 PUFA had a profound inhibitory effect on sodium channels, reducing the peak sodium current by more than 50% and shifting the steady-state inactivation towards negative potentials, thus reducing excitability. This finding was supported by other similar studies [54,55] leading to the hypothesis that n-3 PUFA exert their predominant anti-arrhythmic effect by their inhibitory action on sodium channels. However, when cardiac cells with high membrane incorporation of n-3 PUFA, which was obtained from animals fed a diet fortified with fish oil, were studied, this effect was not consistently reported. Further studies in laboratory animals revealed that n-3 PUFA have a diverse range of effects on other ion channels, such as potassium channels, L-type calcium channels, sodium-calcium exchanger proteins, and calcium-handling proteins as shown in table-5.

Table 5: Summary of animal experiments on the effect of n-3 PUFA on ventricular arrhythmogenesis

Study Type	Authors	Publication details	Main Findings
Dietary supplementation in a whole animal model- Rat	McLennan P.L et al	Am Heart J-1988	Incorporated tuna fish oil reduced vulnerability to both ischemic and reperfusion arrhythmias
Dietary supplementation in a whole animal model- Rat	McLennan P.L et al	Am J Clin Nutr.-1993	n-3 PUFA significantly reduced reperfusion arrhythmias. Fatal ventricular fibrillation (VF) was significantly reduced in the n-3 PUFA group compared with saturated fat.
Dietary supplementation in a whole animal model- Marmoset monkey	McLennan P.L et al	Am J Clin Nutr.-1993	n-3 PUFA reduced vulnerability of normal or ischemic myocardium to arrhythmias (measured as VF threshold) in a nonhuman primate.
Intra venous administration in a whole animal model- Dog	Billman G.E et al	Circulation-1999	Intravenous administration of EPA or DHA prevented fatal ischemia-induced arrhythmias in an infarct model.
Acute application in ventricular myocytes- Rat & Guinea pig	Macleod J.C et al	Eur J Pharmacol-1998	Acute application of EPA resulted in shortening of action potential and increase in relative refractory period.
Dietary supplementation, incorporation in ventricular myocytes- Pig	Verkerk A.O	Cardiovasc Res-2006	Shortening of action potential duration, inhibition of L type calcium current, reduced re-opening of calcium channels at plateau potentials which could prevent triggered activity, no effect on transient outward current (I_{to}) but an increase in I_{k1} and I_{kS} pottasium currents and inhibition of sodium-calcium exchanger current.
Acute application in whole heart preparation- Rabbit	Dhein S	Naunyn-Schmiedeberg's Arch Pharmacol - 2005	Substantial reduction in velocity of impulse conduction (longitudinal) & less so with transverse conduction
Acute application in isolated ventricular myocytes- Rat (Neonatal)	Xiao Y.F	Proc Natl Acad Sci-1995	Substantial (51%) reduction in peak sodium current.
Acute application in isolated ventricular myocytes- Rat (Adult)	Leifert W.R	J Physiol-1999	EPA & DHA shifted the voltage dependence of activation of the sodium channel to more positive potentials & this effect correlated with increase in membrane fluidity
Dietary supplementation- Incorporation in ventricular myocytes- Rat (adult)	Leifert W.R	J Nutr Biochem-2000	Incorporated n-3 PUFA had no effect on peak sodium current or the voltage dependant activation of sodium channels.
Acute application in isolated ventricular myocytes-Guinea pig	Ferrier G.R.	Cardiovasc Res-2002	Suppression of L type calcium current while preserving myocardial function.
Acute application in isolated ventricular myocytes- Rat (Adult)	Bogdanov K.Y.	Am J Physiol Heart Circ Physiol-1998	Inhibition of transient outward potasium current (I_{to})
Acute application in cultured myocytes	Xiao Y.F	Biochem Biophys Res Commun-2004	Inhibition of outward & inward sodium-calcium exchanger current
Acute application in isolated ventricular myocytes- Rat (Adult)	Swan J.S.	Cardiovasc Res-2003	Reduced frequency of spontaneous waves of calcium release (a function of sarcoplasmic reticulum), decreased diastolic calcium concentrations and increased calcium wave amplitudes and propagation
Dietary supplementation, incorporation in ventricular myocytes- Pig	Berecki G	Heart Rhythm-2007	Myocytes from n-3 PUFA treated pigs displayed decreased SR calcium content, reduced L-type calcium current (I(Ca,L)), and less recruitment of the sodium-calcium exchange current (I(NCX)) in response to norepinephrine.
Dietary supplementation- Incorporation Langendorff-perfused hearts- Rabbit	Dujardin KS	Am J Physiol Heart Circ Physiol-2008	DHA inhibited ultra fast sodium current and reduced dofetilide-induced changes in triangulation, reverse use-dependence, instability, and dispersion of cardiac action potential (TRIaD).
Acute application in Vivo- Pericardial administration- Pig	Xiao YF	Am J Physiol Heart Circ Physiol-2008	Pericardial infusion of DHA reduced malignant arrhythmias and infarct sizes in a porcine infarct model
Acute application in isolated ventricular myocytes- Heart failure-Human & Rabbits	Den Ruijter HM	Circulation-2008	n-3 PUFA abolished triggered activity, reduced delayed afterdepolarizations & calcium after-transients, reduced action potential shortening & intracellular calcium elevation in response to noradrenalin

n-3 PUFA have also been shown to alter membrane fluidity[56], with consequent effects on ion transport. Thus, we would expect that the net effect would be derived from the sum of all these effects, on the basis of the relative concentrations and potencies of circulating free and incorporated n-3 PUFA, along with the state of excitability of the substrate and mechanism of arrhythmia initiation.

In addition to a direct anti-arrhythmic effect, other mechanisms that could explain some or all of the observed benefits from large clinical trials have been reported. These mechanisms are: beneficial modulation of the autonomic tone shown as improved heart rate variability;[57–59] reduction in basal heart rate,[60] probably due to an inhibitory effect on the pacemaker current (the funny current-I_f) in the sinus node cells; [61] and nutritional preconditioning similar to ischaemic preconditioning, restricting infarct size and reducing reperfusion-induced arrhythmias. [62] However, in the OMEGA multicentre study [63], no significant reduction in sudden cardiac death or coronary events were reported in a cohort of patients who had an optimum use of conventional therapy, such as β blockers, statins, and angiotensin converting enzyme inhibitors with a high rate of revascularisation procedures, which are used in standard clinical practice. Even though this study had a low power to detect such events, the possibility that n-3 PUFA might not confer additional benefits to those treated with optimum conventional medical therapy needs to be addressed, because previous studies were done in patients with suboptimum use of these agents as shown in table-6.

Table-6: Comparison of concomitant drug therapy and cardiovascular (CV) mortality in randomised trials with n-3 PUFA.

Trial Acronym & Year of publication	Anti-platelet drugs	Cholesterol lowering agents	Beta-Blockers	ACEI/ ARB	Relative risk reduction in CV mortality
DART (1989)	10.2%	NA	29.4%	NA	31%
GISSI-Prevenzione* (1999)	87.9%	28.6%	41.2%	40.9%	17.7%
GISSI HF** (2008)	58%	22.6%***	64.9%	93.5%	7.3%
OMEGA (2010)	95%	94%	94%	83%	No difference

ACEI: Angiotensin convertase inhibitor, ARB: Angiotensin receptor blocker, NA = Data not available, *All data derived from values at 6 month follow-up, when the reduction in CV mortality was significant, ** 50% of patients had non-ischaemic cardiomyopathy, ***open-label use (one of the trial arm randomized to rosuvastatin).

1.3.2: n-3 PUFA in Heart Failure and Arrhythmias

Findings from epidemiological studies[64] have shown an inverse association between consumption of fish and risk of heart failure. In a large observational study with 60 000 participants who were followed up for 13 years, investigators reported a reduction in death attributable to heart failure with increased fish intake. [65] The Atherosclerosis Risk in Communities (ARIC) study, [66] a prospective study of 3592 white men and women, reported for 14.2 years of follow-up that raised serum concentrations of n-3 PUFAs, especially docosahexaenoic acid, were associated with a lowered incidence of heart failure in women. The GISSI Prevenzione Investigators [67] reported that the observed reduction in sudden cardiac death in patients who had had myocardial infarction was most pronounced in those

with evidence of systolic left-ventricular dysfunction. In a large randomized study, GISSI HF Investigators [42] reported reductions in overall mortality and admissions in patients with New York Heart Association class II–IV heart failure with 1 g per day of EPA and DHA. Even though the observed benefit was modest, the fact that the benefits seen were incremental to optimum standard therapy lends support to use of n-3 PUFAs in management of heart failure.

1.3.3: n-3 PUFA and Atrial Arrhythmias

Atrial arrhythmias, particularly Atrial fibrillation (AF) is the most common cardiac arrhythmia reported in clinical practice. In large epidemiological studies [68,69] investigating the effect of fish intake on the risk of development of atrial fibrillation, researchers reported an absence of benefit, but in a prospective population-based study of adults 65 years and older investigators reported that risk of this disorder was lowered with consumption of grilled or baked fish,[70] with a possible dose-response effect that was confirmed by measurements of plasma concentrations of EPA and DHA. In another observational study [71] in men older than 42 years, high serum concentrations of DHA had a protective effect against atrial fibrillation during a follow-up of 17 years.

Data for the role of therapeutic supplementation of n-3 PUFA in management of atrial fibrillation are restricted to one study by Calo et al. [39] This study was conducted as a randomised, open-label study with the use of 2 grams/ day of concentrated n-3 PUFA (Omacor™) for a minimum period of 5 days preceding CABG surgery and evaluated the incidence of new onset of AF following CABG as a primary outcome measure. In this study, 160 patients undergoing cardiac surgery were either treated with standard therapy (79) or standard therapy plus 2g/day of n-3PUFA (81) and the incidence of AF following surgery was evaluated in the pre-operative period by a daily review of stored ECG traces in a bedside

monitor. In this study, post-operative AF developed in 27 patients of the control group (33.3%) and in 12 patients of the n-3 PUFA group (15.2%) ($p = 0.013$). Such beneficial effect was seen even in patients who received supplements for as little as 5 days prior to surgery. The mechanism of action of n-3 PUFA in reducing AF following cardiac surgery is not very clear but the cause and pathology of post-operative atrial fibrillation is unique with a significant role for inflammation in the causation of this arrhythmia.

1.4: Effects of n-3 PUFA on Ion Channels, Exchangers and Calcium Handling Proteins.

1.4.1: Putative Mechanisms of anti-arrhythmic action of n-3 PUFA

A number of plausible mechanisms have been proposed to explain the anti-arrhythmic effects attributed to fish oils: structural, metabolic, autonomic, and electro-physiological. n-3 PUFA satisfy the structural requirements of anti-arrhythmic agents identified by in vitro studies to consist of a long acyl hydrocarbon tail, ≥2 unsaturated carbon-carbon double bonds, and a free carboxyl group at one end. Fatty acids are essential fuels for mechanical, electrical, and synthetic activities of the heart.[72] EPA and DHA are preferentially bound at the *sn*-1 position in storage triglycerides, where lipases are most active and thus are mobilized more rapidly from adipose tissue stores in response to physiologic demand than are saturated fat and monounsaturated fat. n-3 PUFA are not active in triglyceride storage form and require phospholipases to be activated [(73)]. Free fatty acids alter excitability and activity of various cardiac ion channels. The anti-arrhythmic properties of n-3 PUFA likely involve modulation of the biochemical processes underlying fatal ventricular arrhythmias [(74)]. The mechanisms that have been proposed include direct effects on cardiac microsomal calcium/magnesium adenosine tri-phosphatase and voltage-gated ion channels, as demonstrated in cultured

neonatal cardiac myocytes. Also included may be effects on the inositol lipid cycle and cell signaling; on the cell membrane, via modification of membrane phospholipids; on gap junction; or anti-inflammatory effects mediated by eicosanoids. Support for a particular mechanism of action appears to depend on the type of study. While dietary studies support mechanisms mediated by changes in n-3 PUFA or their metabolites in plasma and vascular tissues, studies in isolated animal hearts and cultured neonatal cardiac myocytes support mechanisms related to direct effects on the electrophysiology of the heart. Although human data have not yielded consistent findings on whether n-3 PUFA influence cardiac arrhythmias through autonomic control, they have not ruled out the possibility of additional direct effects on ion channels in cardiomyocytes. Autonomic regulation involves control of inward sodium and calcium currents, which promote depolarization, and the outward potassium current, which opposes depolarization. n-3 PUFA have prevented or attenuated beta adrenergic agonist induced arrhythmias in cultured myocytes in the absence of confounders such as hormones and neurotransmitters.[75]

While multitude of effects have been postulated, the direct effect of fish oils on ion channels (see later) have been extensively explored on animal studies and have yielded variable results based on the type of arrhythmia studied and the species of the animal used.

1.4.2 Acute administration Vs Incorporated Fatty acids

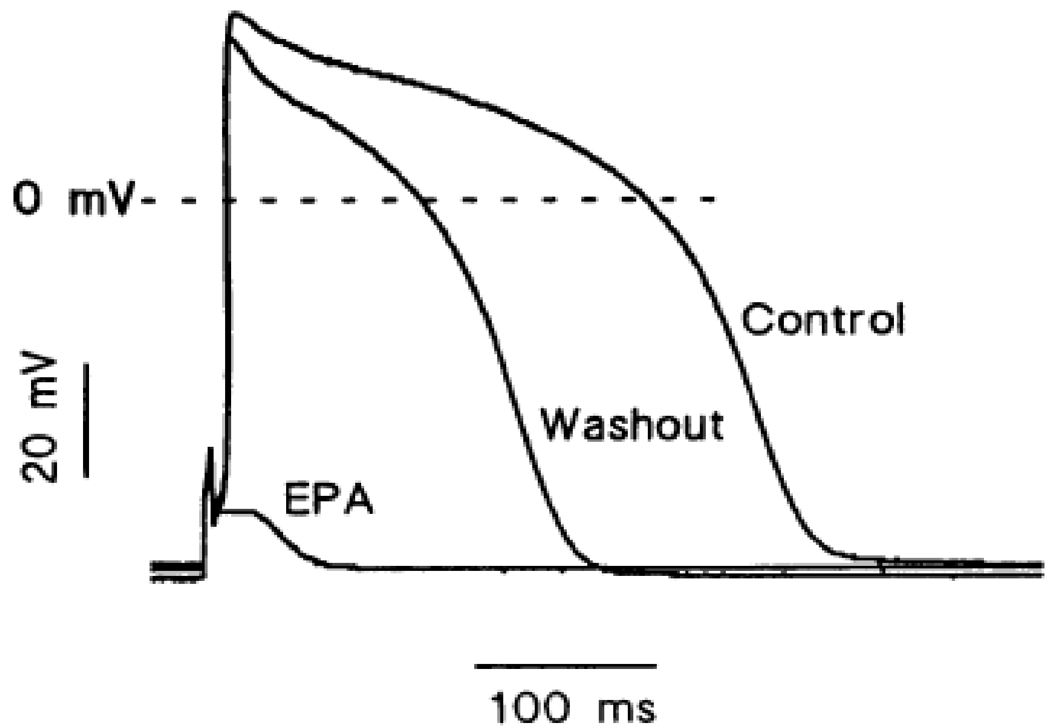
Increased consumption of fish oil leads to high levels of circulating n3-PUFAs in the blood [76] and also increase the amount of n3-PUFA incorporated into the sarcolemma of various tissues[77]. However, data on plasma levels of n3-PUFA after dietary interventions are scarce. In post menopausal women, fish oil supplements (2 g EPA and 1.4 g DHA) for 5 weeks increased plasma concentrations of EPA and DHA up to 0.5–0.7 mM[78]. Animal studies have

shown that feeding a diet rich in n-3 PUFA for a period of about 8 weeks can increase the serum levels of EPA and DHA to 1.0-1.3mM[79]. It is not clear if the circulating fatty acid is indeed free fatty acid as they could be bound to transport proteins (as most fatty acids are) and therefore their ready availability during an arrhythmia provoking situation such as acute myocardial infarction is not certain. Dietary administration of fish oil leads to the incorporation of n3-PUFAs into all the membranes including myocardial membranes of the heart. Several weeks after the start of the dietary intervention with fish oil, n3-PUFAs account for $\sim$25% of total lipids in the sarcolemma. Recent studies have focused on the relative contributions of circulating (presumably free) fatty acids and incorporated n3-PUFA to the beneficial effects on the clinical incidence of arrhythmias and myocardial cellular electrophysiology [80-82] This is particularly important while considering the beneficial effects of "therapy" with fish oil supplements.

1.4.3 n-3 PUFA and cardiac membrane excitability

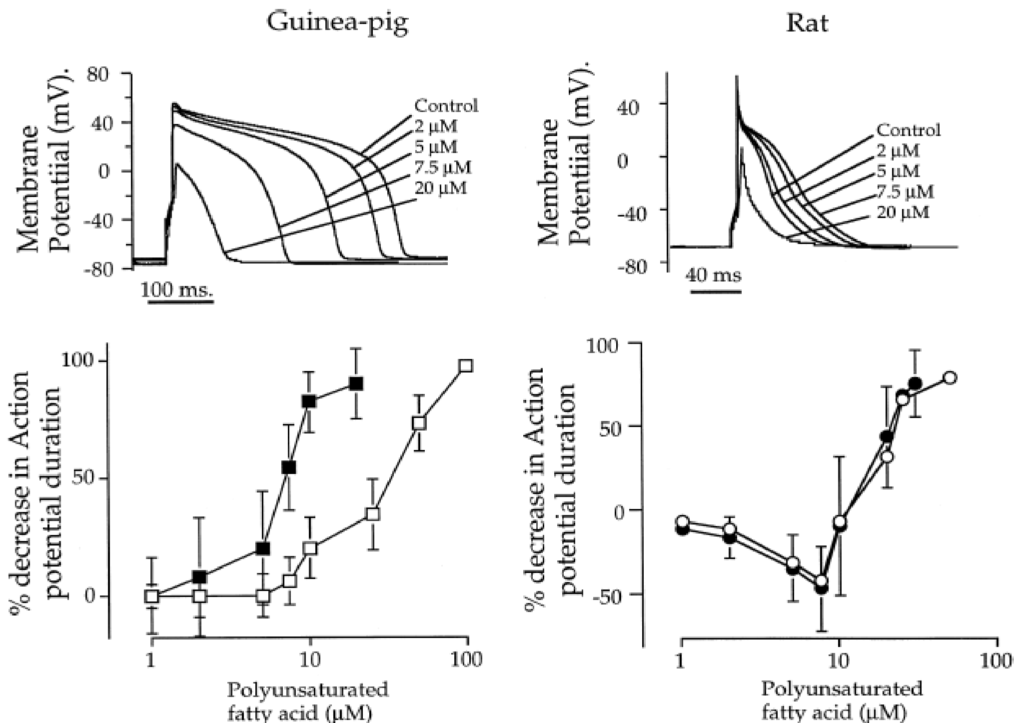
Several studies have indicated that free long-chain polyunsaturated fatty acids (PUFA) EPA and DHA can markedly reduce the contraction rate of isolated, spontaneously beating cardiomyocytes and prevent or terminate the tachyarrhythmias and fibrillation induced by ouabain, high extra cellular calcium and β-adrenergic agonists[83-85]. However, the mechanisms underlying the anti-arrhythmic effect of the fatty acids are far from clear. Kang et al examined the effects of various fatty acids on membrane electrophysiology in neonatal rat cardiac myocytes[86]. They reported that application of free PUFA, but neither monounsaturated nor saturated fatty acids, significantly reduced membrane electrical excitability by increasing the threshold for action potential(more positive), the resting-membrane potential (more negative) and the refractory-period duration as shown in figure-1.2.

Figure 1.2: Effect of EPA on action potential generation in response to a fixed depolarizing impulse. Exposure to 10 µM EPA failed to elicit an action potential for the same depolarizing current and washing with BSA regenerated the action potential (Modified from Kang et al[85]).



Whilst such studies seem to indicate that there is a straightforward inhibitory effect on the electrical excitability of cardiac myocyte membranes, this effect is far from clear or straightforward. Other studies [87] have shown that this inhibitory effect is variable at different concentration levels and the optimal concentration to obtain an inhibitory effect depends on the species studied as shown in figure-1.3.

Figure 1. 3: Records of action potentials from a guinea-pig (left) and rat (right) ventricular myocyte showing control action potential and the effects of the addition of 2, 5, 7.5 and 20 mM EPA. The effects of DHA are shown as open symbols and EPA acid as filled symbols.



The plot shows a reduction in the action potential duration of guinea-pig cells but a prolongation of the rat action potential duration at low concentrations and a reduction in rat action potential duration at concentrations above 10 mM. (Modified from Macleod JC at al[99])

Such inhibitory effects on membrane excitability are often achieved by direct effects on the ion channels responsible for the initiation and conduction of an action potential. Hence the effects of n-3 PUFA on various cardiac ion channels have been studied on various animal models as detailed below.

1.4.4: n3-PUFA and the Sodium Current

The cardiac sodium current (I_{Na}) is responsible for the upstroke of the action potential and plays an important role in impulse conduction. Studies on animal cells and cultured human cells have shown that the peak I_{Na} was significantly reduced after acute administration of EPA and DHA (5–10 μM) [88,89]. In addition, the steady-state

inactivation of the current is shifted towards more negative potentials, without any significant change in activation properties[90,91]. EPA also enhanced slow inactivation and markedly prolonged recovery from inactivation of human cardiac sodium channel-hH1α[89] Generally, n3-PUFAs showed higher affinity to block channels that were in the inactivated state compared to channels in closed or resting state[89]. Interestingly, substitution of a single amino acid, asparagine with lysine at site 406 (N406K) in the D1-S6 region of hH1α reduced the potency of EPA to inhibit I_{Na} . This has been taken as evidence that n-3 PUFA interact directly with the ion channel protein[92].

As mentioned above, some studies have shown that acute application of n-3 PUFA does not affect the activation properties of the sodium channel[88,89]. However, in adult rat ventricular myocytes, acute administration of n3-PUFA shifted the voltage dependence of activation of the cardiac sodium channel to more positive potentials[93]. In this study, the effects of EPA and DHA on the cardiac sodium channel correlated with their ability to increase membrane fluidity[93]. This variability highlights the species variability in the effect of free n-3 PUFA on voltage dependent sodium channels.

In contrast with the acute effects due to direct application of free fatty acids, peak sodium current ($I_{Na)}$) was unaffected by incorporated n3-PUFA in ventricular myocytes isolated from animals fed a diet rich in fish oil[94,95]. In these studies voltage dependence of activation remained unaltered, whereas a shift in inactivation towards more negative potentials was observed[94,95].

In summary, acutely administered n3-PUFA reduces I_{Na}[88,89,93]. This contributes to the observed reduction in excitability and to the slowing of ventricular conduction[96,97]. However, incorporated n3-PUFAs do not alter I_{Na} [94,95].

1.4.5: n-3 PUFA and the re-polarizing currents

Early rapid repolarization or the notch of the action potential (phase 1) is caused by the transient outward current, carried by potassium ions (I_{to1}) and/or chloride ions ($I_{Cl(Ca)}$ or I_{to2}). The slow and rapid components of the delayed rectifier current (I_{Ks} and I_{Kr}, respectively) are responsible for rapid repolarization (phase 3) of the action potential. The inward rectifier current (I_{K1}) contributes to the terminal phase of repolarization and to the maintenance of the resting membrane potential.

The Kv4.3 gene encodes a large proportion of the ion channel responsible for I_{to1} and the n-3 PUFA, DHA has been shown to block the Kv4.3 current in a concentration dependent manner in a stable transfected mammalian cell line[(98)]. I_{to1} has also been shown to be inhibited following acute administration of EPA and DHA (IC_{50} < 10 µM) in rat and ferret ventricular myocytes[(99,100)]. In the presence of the antioxidant alpha-tocopherol, this effect seems to be less pronounced, but still significant in rat ventricular myocytes[101]. However, incorporated n3-PUFAs did not alter I_{to1} in ventricular myocytes isolated from rats fed a diet rich in fish oil[(94)].

I_{to} is larger in subepicardial ventricular myocytes compared to mid- and endomyocardial ventricular myocytes and thereby contributes to transmural dispersion in repolarization[102]. Block of I_{to1} would be expected to attenuate transmural dispersion of repolarization and thereby prevent re-entrant tachyarrhythmias[(102)] but so far the effects of fish oil on transmural dispersion of repolarization have not been investigated.

I_K has been shown to be decreased following acute administration of EPA and DHA in ferret cardiomyocytes[(103)]. I_K consists of two components I_{Kr} and I_{Ks}. Acutely administered DHA blocked the human *ether-a-go-go*-related gene (*HERG*) channel which encodes the pore-forming subunit of the ion channel carrying I_{Kr} in a time-, voltage- and

use-dependent manner[104]. However, incorporated n3-PUFAs did not cause any change in I_{Kr} of pig ventricular myocytes[94].

I_{Ks} has been studied in Xenopus oocytes upon expression of the channel pore-forming subunit, KvLQT1, in the presence or absence of the auxiliary subunit, hminK. I_{Ks} was enhanced by acute administration of DHA but not by EPA[105]. Upon incorporation of n3-PUFA, I_{Ks} was increased in pig ventricular myocytes[94].

A decrease of I_{Kr} following acute administration of n3-PUFA may, at least in part, explain why n3-PUFA prolong the action potential as less repolarizing current is present during the repolarization phase of the action potential. On the other hand, augmentation of I_{Ks} by acutely administered n3-PUFA leads to increased repolarizing current during the repolarization phase of the action potential. Whether the observed changes in repolarizing potassium currents caused by n3-PUFA lead to action potential prolongation or shortening will largely depend on the delicate balance between these and other depolarizing and repolarizing currents, species-differences regarding channel protein expression and the concentration of free EPA and DHA.

I_{K1} did not change upon acute administration of n3-PUFA in ferret cardiomyocytes[103]. Incorporation of n3-PUFA resulted in an increase of I_{K1} in ventricular myocytes isolated from pigs fed a diet rich in fish oil[94]. Increased I_{K1} by incorporated n3-PUFA contributes to the observed action potential shortening[94]. In addition, an increase in I_{K1} may decrease excitability and thereby reduce DADs and triggered activity.

The transient outward current is not always carried by potassium ions but is, in several species, carried by chloride ions ($I_{Cl(Ca)}$)[106]. Data on $I_{Cl(Ca)}$ following acute administration of n3-PUFA are lacking. Incorporation of n3-PUFA into ventricular myocytes left $I_{Cl(Ca)}$ unaltered[94]. Other currents that affect action potential shape and duration, e.g. ultra-rapid

delayed rectifier K^+ current, have not yet been investigated in the presence of (circulating or incorporated) n3-PUFA.

In summary: (1). I_{to} is inhibited by acute administration but not affected by incorporated n3-PUFAs. (2). I_{Kr} is inhibited by acute administration and not altered by incorporation. Inhibition of I_{Kr} can lead to prolongation of action potential. (3). I_{Ks} is enhanced both by acute administration and by incorporation. (4). I_{K1} is not altered by acute administration but enhanced by incorporation. This would have the effect of shortening of the action potential.

In a clinical setting, following supplementation with n3-PUFA for a particular length of time, we can assume that the effects of incorporation would be reproducible. However, the availability of free fatty acids in the plasma (akin to acute administration of FFA) depends on various factors that come into play in vivo, the most important of which is likely to be the activity of phospholipases essential for the release of free fatty acids.

1.4.6: n3-PUFA and the calcium current

It is known that the L-type calcium current ($I_{Ca,L}$) is the depolarizing current responsible for the plateau phase of the action potential and contributes to the duration of the ventricular action potential. Acute application of the n-3 PUFA on isolated animal ventricular cells (neonatal rat, adult rat and guinea pig) showed that $I_{Ca,L}$ was suppressed in a concentration-dependent manner ($IC_{50} < 10\ \mu M$) [107,108,109]. Activation properties of $I_{Ca,L}$ remained unchanged, and a negative shift of steady-state inactivation was observed in these studies. Interestingly, n3-PUFA reduced $I_{Ca,L}$, while preserving myocardial function [109].

Incorporation of n3-PUFAs into the sarcolemma also reduced $I_{Ca,L}$ significantly while leaving activation properties unaltered[94]. At plateau potentials, 'reopening' of the L-type calcium channel was reduced in ventricular myocytes with incorporated n3-PUFAs compared to the control myocytes[93]. This may prevent early after depolarisations (EAD) and thereby triggered activity[94]. Thus, acute administration of n3-PUFAs to ventricular myocytes reduces $I_{Ca,}$[108,109] and thereby lowers the plateau of the action potential. Incorporated n3-PUFAs also reduces $I_{Ca,L}$ and, more importantly, inhibits 'reopening' of the calcium channel at plateau potentials.

1.4.7: n-3 PUFA and the sodium-calcium exchanger–(NCX)

The sodium-calcium exchanger (NCX) exchanges 3 sodium ions for 1 calcium ion and is therefore electrogenic. It can generate inward or outward current and contributes to the shape and duration of the action potential. Outward and inward sodium-calcium exchanger current (I_{NCX}) was inhibited by acute administration of n3-PUFAs in HEK293t cells[110].

Leifert et al. showed that a diet rich in fish oil increased the time constant of decay of Ca^{2+} transients in response to caffeine in isolated rat cardiomyocytes and suggested that NCX was involved[111]. This finding was supported by experiments in ventricular myocytes, where both outward and inward I_{NCX} were reduced in the presence of incorporated n3-PUFA[94].

Inhibition of I_{NCX} results in action potential shortening because less depolarizing current is available during the final repolarizing phase of the action potential[94,110]. Furthermore, I_{NCX} contributes to DAD formation after spontaneous sarcoplasmic reticulum calcium release. Possibly, reduced I_{NCX} by both acute administration and incorporated n3-

PUFAs reduce DADs and triggered activity. However, such an inhibitory effect has not been reproduced by other workers. A study by O`Neill et al[112] using the measurement of trans-sarcolemmal calcium fluxes and intracellular calcium in rat ventricular myocytes showed that the application of EPA resulted in a significant decrease in the frequency of spontaneous calcium waves. The majority of this was due to inhibition of the Calcium release mechanism. In addition they also showed that EPA did not alter the rate of fall of calcium in the caffeine response (used as an indicator of surface membrane calcium efflux pathway activity). Thus, they concluded that the lower resting level of calcium observed in EPA is due to a lower influx of calcium across the surface membrane rather than increased activation of efflux pathways. This and other studies suggest that the calcium regulation mechanism rather than the NCX may be altered by PUFAs.

1.4.8: n-3 PUFA and calcium regulation

Intracellular calcium handling plays an important role in the genesis of triggered activity[113]. In experiments on rat ventricular myocytes, acute administration of EPA reduced the amplitude of calcium transients and calcium sparks without modifying calcium spark kinetics[114]. The frequency of spontaneous waves of calcium release was also diminished in the presence of EPA. This indicates that sarcoplasmic reticulum function is affected by acute administration of EPA. Additionally, EPA decreased diastolic calcium concentrations and imaging of calcium waves showed that EPA also increased calcium wave amplitudes and propagation velocity [115]. Increased calcium wave amplitudes by EPA correlated with enhanced sarcoplasmic reticulum load in rat ventricular myocytes[116-117]. It could therefore be concluded that n3-PUFA reduce sarcoplasmic reticulum calcium uptake and also inhibit calcium release. The potency of n3-PUFA to reduce open probability (P_o) of RyR was demonstrated in isolated sarcoplasmic reticulum vesicles[116,118].

Spontaneous calcium release from the sarcoplasmic reticulum is the underlying mechanism for DAD-related arrhythmias in heart failure(119). Increased diastolic calcium levels have been shown to induce spontaneous calcium releases from the sarcoplasmic reticulum in a rabbit model of heart failure(120). In that model, diastolic and systolic $[Ca^{2+}]_i$ levels were reduced after acute administration of EPA to isolated ventricular myocytes. Furthermore, spontaneous calcium releases and DADs were reduced by EPA after burst-pacing in the presence of noradrenaline (121). In contrast, incorporated n3-PUFA did not cause any alterations in diastolic calcium or calcium transient amplitude in pig ventricular myocytes. In these animals, the duration of the calcium transient was shortened probably secondary to the shorter action potential recorded in these myocytes(94). Similar results were obtained in ventricular myocytes isolated from rats fed a diet rich in fish oil where calcium transients and diastolic calcium values remained unaltered in the presence of incorporated n3-PUFAs. In this study, sarcoplasmic reticulum calcium content was also unaffected by incorporated n3-PUFAs(122).

In summary, the effects of acute administration of n3-PUFAs on intracellular calcium handling are different from those with incorporated n3-PUFAs. Acute administration of n3-PUFAs leads to a decrease in diastolic calcium concentration in rat ventricular myocytes and in ventricular myocytes isolated from rabbits with heart failure (115,117,121). Furthermore, EPA reduces spontaneous waves of calcium release from the sarcoplasmic reticulum that underlie DAD-related arrhythmias (121). Therefore, acute administration of n3-PUFAs may reduce triggered activity based on spontaneous calcium releases and DADs.

1.4.9: n-3 PUFA and expression levels of cardiac ion channel proteins

There is clear evidence that n-3 PUFA supplementation readily results in a significant incorporation on the cardiac cell membranes altering the composition of the membrane phospholipids. There is also evidence that n-3 PUFA influence the function of multiple cardiac membrane bound ion channels and may alter the fluidity of the membrane itself. However, to date, there is very little evidence about the effect of n-3 PUFA incorporation on the expression levels of any of the membrane bound proteins that influence cardiac arrhythmogenicity. Recent work by Guizy M et al[(123)] has reported that n-3 PUFA may exert a direct inhibitory effect on cardiac Kv1.5 ion channels without affecting the expression levels of these channels while Sarazzin et al [(124)] have reported a significant change in the expression levels of cardiac connexins (inter cellular communication channels) in a canine model of atrial fibrillation.

1.5: AF and cardiac calcium handling

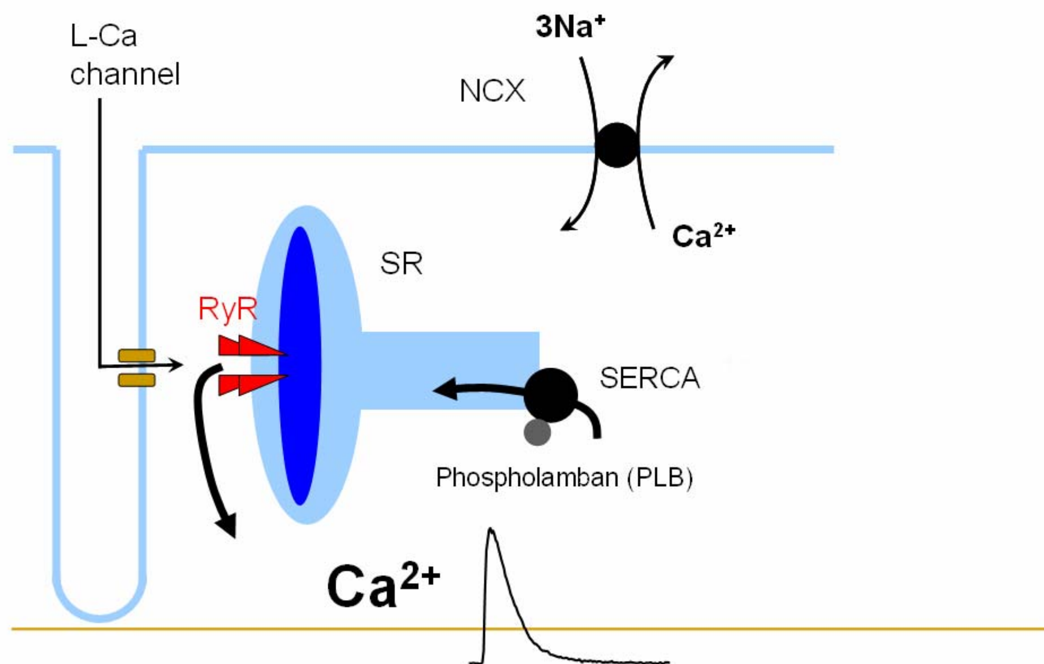
Recent evidence indicates that altered intracellular calcium handling is an important pathophysiological mechanism in the initiation and sustenance of AF [(184)]. It has been reported that increase in the atrial activation rate and the subsequent initial $[Ca^{2+}]_i$ overload seen in AF leads to further 'remodelling' of intracellular calcium handling. There is considerable interest in unravelling the contribution of altered intracellular calcium handling to different types of AF. It is also noted that changes in intracellular calcium homeostasis preceding the onset of AF, in conditions which predispose to AF such as heart failure, appear to be different from changes in calcium handling developing after the onset of AF. Such alterations in intracellular calcium handling could have significant effect on three specific aspects of AF pathophysiology, (i) excitation–

transcription coupling and calcium dependent signalling pathways, (ii) atrial contractile dysfunction, and (iii) arrhythmogenicity [184].

1.5.1: Mechanisms involved in cardiac calcium cycling

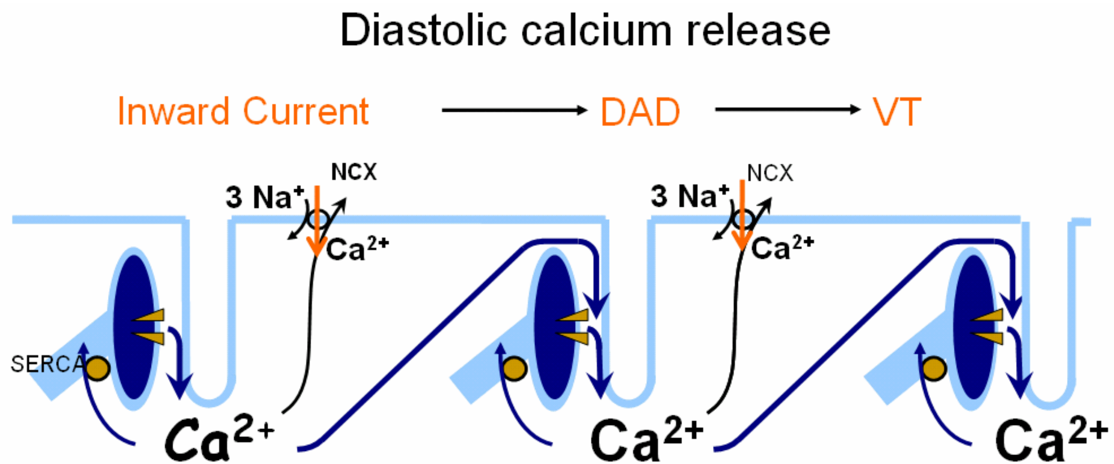
Activation of voltage-gated L-type calcium channels by membrane depolarization leads to the influx of a small amount of calcium into the cardiac myocyte, which activates the release of larger quantities of calcium from the nearby calcium stores in the sarcoplasmic reticulum (SR). This amplification of the trigger calcium and its propagation results in a cell-wide transient increase in $[Ca^{2+}]_i$ that initiates contraction as free calcium binds to the myofilaments. This calcium induced calcium release (CICR), which is characteristic for cardiac excitation–contraction (EC) coupling, is fundamentally the same in atrial and ventricular myocytes [185]. Calcium homeostasis is restored and relaxation occurs as calcium is released from the myofilaments, pumped back into the SR by the SR Ca^{2+} ATPase and extruded from the cell by the sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX) as shown in figure 1.2.

Figure 1.4: Mechanisms involved in cardiac calcium cycling



1.5.2: Diastolic calcium release and pro-arrhythmias

In some disease conditions such as heart failure where there is a calcium overload in the SR, during diastole, small amounts of calcium is released locally from the SR which then leads to a small depolarising current due to the effect of the electrogenic sodium calcium exchanger pump which extrudes calcium from the cytosol. For each molecule of calcium extruded 3 molecules of sodium enter the cell and thus becomes electrogenic. When this propagates along the cell, the amount of calcium released increases exponentially due to the phenomenon of calcium induced calcium relase as described above and eventually a depolarising wave ensues which is termed delayed after depolarisation or DAD. Such diastolic depolarisations have been shown to cause ventricualr arrhythmias in patients with heart failure. There is increasing evidence that a simialr mechanism is active in the intiation of atrial fibrillation [186, 187].



1.5.3: Alteration in SR function and arrhythmogenic diastolic calcium release in AF

As discussed above, alterations in claium handling and diastolic calcium release seem to play an important role in the initiaiton of AF. In that respect, there are two distict patterns of change in the cardiac calium handling seen in animal models of AF. In the rapid ventricular pacing-induced canine heart failure model of atrial fibrillation, it has been reported that SR calcium load is increased [(186)] and phosphorylation of PLB was increased, compatible with increased SR calcium reuptake. It has also been shown in the same model that RyR2 protein expression was reduced [(186)]. However, in goats with chronic atrial dilatation, SR calcium load was decreased [(188)]. In right atrial myocardium from dilated goat atria, PLB phosphorylation was reduced, which is compatible with decreased SERCA2a function. In addiiton, in the same model, hyperphosphorylation of RyR2 , a mechanism that is thought to increase the channels' open probability was also seen [(189,190)] consistent with diastolic Ca^{2+} 'leak' from the SR. Thus, in chronic atrial dilatation in goats, reduced SR Calcium load might be due to increased loss and reduced reuptake of calcium into the SR due to reduced SERCA activity and increased open probability of RYR2.

1.5.4: Effect of n-3 PUFA on cardiac calcium regulation

Cellular experiments have consistently shown that acute administration of n3-PUFAs leads to action potential shortening resulting from its inhibitory effect on ionic currents such as sodium current (I_{Na}) and L-type calcium current ($I_{Ca,L}$) [99,100,108] A shorter action potential prevents calcium overload and DADs as a result of the longer diastolic interval and increased time for calcium removal [191]. Furthermore, acute application of n-3-PUFAs to normal myocytes also decreases calcium transients, inhibits calcium sparks, and reduces spontaneous calcium release from the SR [112,115]. Recently Den Ruijter et al reported that acute application of EPA and DHA to isolated ventricular myocytes from a rabbit model of heart failure and human explanted (heart failure) heart shows that there is a significant reduction in both systolic and diastolic levels of calcium with evidence of inhibition of triggered activity. This effect could have a significant anti-arrhythmic property in heart failure.

1.6: Summary of current evidence

Animal experiments and clinical studies have reported that n-3 PUFA may have a protective effect against life threatening ventricular arrhythmias following myocardial infarction and heart failure. However, the effect of n-3 PUFA on atrial arrhythmias is less well understood. There is some suggestion that n-3 PUFA may protect against AF following CABG but this data is derived solely from one open-label clinical study, which by nature of the study design allows for a potential for bias. There is considerable volume of data from animal studies showing that n-3 PUFA have a multitude of effects on cardiac ion channels all of which could be potentially anti-arrhythmic but the mode of action of n-3 PUFA to achieve such an effect is still unclear. There is very little data on the influence of membrane incorporation of n-3 PUFA

on the expression levels of these ion channel proteins. Moreover, the clinical benefit of reduced risk of sudden death has been shown predominantly following an MI and in patients with heart failure. In both these situations, the primary arrhythmogenic mechanism is alteration in cellular calcium handling. Recent evidence suggests that altered calcium homeostasis may have a role in the initiation of atrial fibrillation. However, the effect of n-3 PUFA on cardiac calcium handling has not been adequately investigated. Hence this study will focus on the effect of membrane incorporation of n-3 PUFA on atrial arrhythmias and the cellular effect on calcium handling proteins in humans.

1.7: Research hypotheses

- Oral supplementation of purified n-3 PUFA (Omacor™) (presented as 1g capsules containing 88% n-3 PUFA) to patients undergoing coronary artery bypass graft (CABG) surgery will lead to a measurable change in the membrane composition of fatty acids in cardiomyocytes and would influence the incidence of atrial fibrillation following coronary artery bypass surgery.
- Incorporation of n-3 PUFA on cardiomyocytes of the atrial appendage tissue (obtained during CABG surgery) will lead to changes in the expression levels of calcium handling proteins and/or other ion channels and transporters. This alteration in expression will lead to a measurable change in the function of the relevant protein.
- These changes could be initially evaluated by the use of quantitative PCR technique and based on the results of this less specific but more sensitive technique (used as a form of screening technique) a focused evaluation of the relevant protein/s can be performed to elucidate the effect of n-3 PUFA on cellular calcium handling and/or other ion channels and transporters in humans.

Chapter 2

CHAPTER-2: MATERIALS AND METHODS

2.1: Clinical methods

2.1.1: Study design

This study was designed as a single-center, randomized, double-blind, placebo controlled clinical trial carried out in the department of cardiology and cardiothoracic surgery in University Hospital of South Manchester (UHSM) Wythenshawe, Manchester. The study was primarily designed to test the hypothesis that n-3 PUFA supplementation would reduce the risk of AF after CABG. The major limitations of previous studies evaluating various interventions to reduce the risk of AF following cardiac surgery and most clinical studies evaluating the anti-arrhythmic potential of n-3 PUFA are the lack of robust monitoring for AF in the former and the lack of objective evidence of levels of n-3 PUFA in the latter, along with a possible confounding effect of dietary PUFA intake on these levels. We sought to address these issues by means of continuous monitoring of AF in the postoperative period for 5 days and by quantifying serum, peripheral blood mononuclear cell membrane and tissue levels of n-3 PUFA to assess if therapy had indeed altered the levels in these cell membranes, along with a food frequency questionnaire to estimate the dietary intake of n-3 PUFA. Such direct estimation of cardiac tissue levels of n-3 PUFA and direct correlation with clinical outcome has not been done in any of the previous clinical studies on this subject. We also estimated serum levels of C-reactive protein (CRP) as a marker of systemic inflammation because systemic inflammation has been suspected to play a role in the initiation of AF after CABG(21,125) and n-3 PUFA have been shown to possess anti-inflammatory properties(126).

The study protocol was approved (under the title- How does fish oil supplementation reduce risk of atrial fibrillation following coronary bypass surgery?- A cellular study) by the

Medicines and Healthcare Regulatory Agency of the United Kingdom and registered with the European Clinical Trials database (EudraCT) as a clinical trial of an investigational medicinal product (Appendix-1). The conduct of the study was approved by the Trent Multi-center Research Ethics Committee (Aappendix-2). The study was sponsored and approved by the R&D department of the University Hospital of South Manchester, Wythenshawe (Appendix-3). The clinical trial was supervised by Dr. Neil C Davidson, Consultant Cardiologist, UHSM, Wythenshawe, as the principal investigator. A subsequent substantial amendment was made and approved by the Trent ethics committee to enable changes to the cellular aspect of the study to be made (Appendix-4). A data monitoring committee was formed consisting 2 independent cardiologists and a statistician from within our institution with a view to monitoring data at 18 months of the conduct of the study and at study completion.

2.1.2: Sample size calculation

The average incidence of AF after CABG in UHSM, Wythenshawe is approximately 50%, which is within the range of previously reported values(21,22). Hence, sample size was calculated with an expected incidence of postoperative AF of 50% in the control group and a relative risk reduction of 55% by n-3 PUFA in accordance with the study by Calo et al(39). This gave a sample size of 54 patients in each group (108 in all), with 80% power to detect a significant difference using simple χ^2 test with the conventional 5% significance level.

2.1.3: Patient recruitment

Inclusion Criteria:

- All patients over the age of 18 years, who were to undergo elective isolated CABG on cardiopulmonary bypass at University Hospital of South Manchester, UK.

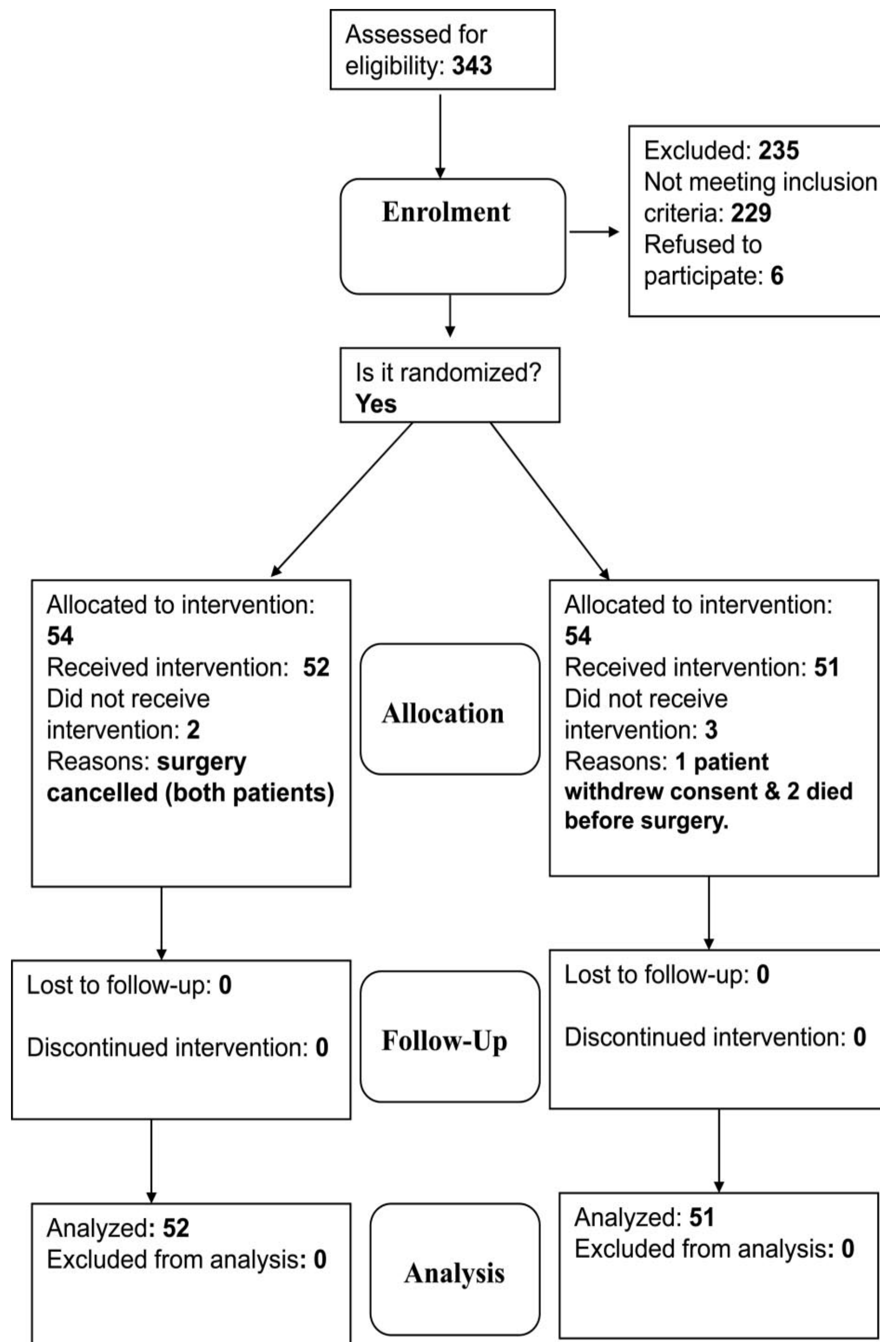
Exclusion Criteria:

- Patients with previous or current history of any atrial arrhythmia.
- Patients who were taking any class 1 or class 3 anti-arrhythmic drugs (Vaughan-Williams classification)
- Patients who were taking or had been taking within the previous 3 months fish oil supplements.

Screening and Enrolment: Patients were identified by screening the cardiac surgical waiting list and those found eligible were sent a copy of the patient information leaflet (version-4) Appendix-5) along with the appointment letter for their pre-admission clinic. This enabled the patients to read through the details of the study and gave them adequate opportunity to raise any queries that they might have when meeting the researcher in person prior to study enrollment. Patients, who were willing to participate in the study, were enrolled during a preadmission clinic visit, which typically took place 1 to 3 weeks before surgery. At enrolment, all participants gave written informed consent to take part in the study. A copy of the consent form is shown as Appendix-6.

Study enrolment commenced in June 2007 and was completed in January 2009. A total of 108 patients were enrolled of 343 eligible patients as shown in Figure-2.1. Consecutive patients were randomly assigned, in a double-blind fashion, and were advised to take 2x1g capsules of (2 g/d) a commercially available n-3 PUFA preparation (Omacor; Pronova Biopharma, Lysaker, Norway) providing 85% to 88% EPA+DHA as ethyl esters and in a ratio of 1.2:1 in addition to standard care (active treatment group) or 2x1g capsules (2g/d) placebo (olive oil) along with standard care (placebo group). Both n-3 PUFA and placebo were presented in identical 1-g capsules. Random assignment was based on a computer generated randomization list obtained using blocks of size 4.

Figure-2.1: Clinical Trial Flow Chart



2.1.4: Venous blood sample collection and processing

Upon enrollment and random assignment, a peripheral venous blood sample was collected in heparinised vacutainers for preparation of serum and PBMC. Whole blood was collected in two 10mls vacutainers (one with heparin for PBMC separation) and one of them

centrifuged at 10000 rpm for 10 minutes to separate serum. The supernatant serum was then transferred into an eppendorf tube using a sterile Pasteur pipette. The other blood sample was used to separate PBMC using Ficoll gradient. The method of separating PBMC is briefly as follows: Two 20ml test tubes were filled with 10mls of Ficoll-Paque Plus solution each. 10 ml of heparinised blood was gently pipetted on top of Ficoll-Paque Plus and centrifuged for 20 minutes at 2000 rpm (no brake**).** The two white blood cell ring fractions were transferred to a new 20 ml test tube using a sterile Pasteur pipette and the volume was adjusted to 10 ml by adding PBS which was again centrifuged at 1700 rpm. The supernatant was discarded the pellet of PBMC was re-suspended in 3 ml of PBMC which was transferred to Eppendorf tubes. Both the serum and PBMC samples were labelled with anonymised identifier code numbers and stored at -80°C for future analysis.

2.1.5: Echocardiographic assessment

All patients had a 2D echo-cardiographic evaluation for left ventricular (LV) systolic function and left atrial (LA) size, using standardized measurements. Patients were deemed to have LV dysfunction if they had an LV ejection fraction (modified Simpson's method) ≤55% and significant atrial dilation if the atrial AP diameter was ≥2.3 cm/m^2 body surface area.

2.1.6: CABG surgery and tissue processing

All participants had CABG surgery using a midline sternotomy incision on cardiopulmonary bypass. On the day of surgery, all patients had a second venous blood sampling and serum and PBMC was prepared as before. During surgery, a small sample of tissue from the right atrial appendage was obtained. This tissue was immediately washed free of blood and dissected into 3 pieces and 2 of the 3 pieces were immediately snap frozen by immersion in liquid nitrogen, labelled with anonymised sample identifier

codes and stored at -80°C for future use in molecular biological studies. The third piece of tissue was immediately processed by the researcher in an attempt to isolate human atrial cells with a view to performing electropysiological studies as detailed below.

2.1.7: Dietary intake of n-3 PUFA

On recruitment, all patients were provided with a self-administered food frequency questionnaire listing commonly consumed food items that are considered to be sources of marine n-3 fatty acids (Appendix-7). All portions were clearly defined and when participants had consumed but less than a defined portion they could choose to enter half a portion so that an accurate estimate of the dietary intake of n-3 PUFA was available for all participants at baseline. Based on the information provided in this questionnaire, their weekly intake of n-3 PUFA was estimated using pre-defined values that reflect the quantity of n-3 PUFA present in a portion of a specified food item. All 103 patients who completed the study provided a valid response to this questionnaire based on which a reliable estimate of the weekly intake of n-3 PUFA in all the study participants was calculated at baseline. All study participants were advised not to change their dietary habits during the study period.

2.1.8: Post-operative arrhythmia monitoring

A heart rhythm monitor (Lifecard CF digital Holter recorder; Space Labs Healthcare, Wash) was attached to the patient's chest in the immediate post-operative period in the cardiac intensive care to continuously record 2-lead ECG data for 5 postoperative days. If patients stayed in the hospital for longer than 5 days, a daily ECG was performed along with a daily clinical follow-up by the researcher. On completion of recording, data from the Lifecard monitor was downloaded onto a Delmar Reynolds computer system (Space

Labs Healthcare) and archived to a disk. At the end of the study, these data were analyzed by 2 independent experts (Researcher and a senior ECG technician) using an automated arrhythmia diagnostic system (Pathfinder Digital; Space Labs Healthcare).

The system divides the continuous ECG recording into 24-hour segments. Briefly, the methods of AF identification were as follows: ECG recordings were loaded and appropriate program sensitivity and detection were checked. Interpretation of whether any arrhythmia existed on each day was performed by analysis of peaks/troughs of the 24-hour trend graphs of heart rate, N-N intervals (the time intervals between consecutive normal beats, reflecting underlying sinus rhythm), and sNN50 (number of pairs of adjacent R-R intervals differing by ≥50 ms). If no arrhythmia was detected, it was recorded as 0 incidence of AF and 0% of recording spent in AF. If the interpretation was inconclusive or if an arrhythmia was thought to have occurred, then the entire 24-hour recording was scanned at an appropriate speed and episodes of AF ≥30 seconds were manually identified and appropriately labeled. The program then calculated the number of AF episodes marked and the percentage of recording in AF. All 12-lead ECGs were manually analyzed by the researcher.

2.1.9: Therapy, monitoring and follow-up after CABG

Patients continued to take n-3 PUFA or placebo in the postoperative period until discharge from the hospital. If patients were unable to take the capsules orally (3 patients in the active treatment arm and 2 patients in the control arm) in the immediate postoperative period, the contents of the capsules were mixed with the naso-gastric feed; this was carried out exclusively in the intensive care unit by the nurse in charge of patient care, without compromising the blinding process. A further blood sample was collected

on the 3rd postoperative day (48 to 72 hours after completion of surgery); serum was prepared and used to measure concentrations of CRP.

Formal participation in the study ended at hospital discharge. However, any hospital admission in the 4 weeks after surgery was evaluated for potential study-related complications.

2.1.10: Clinical outcome measures

The primary outcome measure was any AF ≥30 seconds in the Lifecard monitor recordings. Secondary outcomes were clinically recognized AF (as documented by the treating clinicians in the patients clinical records), AF burden (defined as the percent of time a given patient is in AF), length of stay in the hospital, and length of stay in the intensive care or high-dependency care unit. In addition CRP levels were measured to assess influence of n-3 PUFA on inflammatory markers and consequent effect on the incidence of AF following CABG surgery.

2.1.11: Clinical indices influencing risk of arrhythmias

There are several ECG markers which have been reported to be useful in predicting the risk of development of atrial and ventricular arrhythmias. One of the consistent parameter that has been associated with a risk of developing atrial arrhythmia is P-wave dispersion (PWD) in a standard 12 lead surface ECG. This is the difference between the maximum and minimum P-wave duration measured in all the 12 leads. It has been suggested that PWD is a measure of the degree of heterogeneity in conduction in the human atrium and larger PWD may pre-dispose an individual to higher risk of atrial arrhythmias [(192)]. Pre-operative PWD has been reported to be useful in predicting post operative AF following CABG. In addition, n-3 PUFA have been shown to alter expression levels of connexins in

canine atrium and may have an influence on PWD. Hence we measured this variable in all the participants pre- and post supplementation. Another important ECG variable that predicts risk of ventricular arrhtymia is heart rate variability (HRV). HRV is a measure of the degree of autonomic tone with lower HRV indicating poor autonomic balance and higher risk of arrhythmias. n-3 PUFA have been reported to influence the autonomic nervous system in a favourable way with lower resting heart rate and an improved HRV particularly in diabetic subjects. Hence we also measured HRV in this study-following n-3 PUFA supplementation- on day 3 following CABG.

1) P-wave dispersion (PWD) in 12 lead ECG: Standard supine 12 lead ECGs (filter range, 0.15 to 100Hz, AC filter 60Hz, 25mm/s, 10mm/mV) were obtained for all patients at recruitment (baseline) and the day before surgery (post supplementation). The effect of n-3 PUFA supplementation on some recognised markers of atrial arrhythmias was evaluated by analysing these ECGs by the following method. All ECGs were scanned and magnified 200% to fit the computer screen. A digital calliper was used to make accurate measurement of the relevant intervals. 2 independent observers (the researcher and a senior ECG technician) reported on these values. Both observers were blinded to the therapy (n-3 PUFA or Placebo) that the patients received at the time of analysis. Reports on P wave duration on each lead for a given 12 lead ECG was generated by each observer by taking an average of 3 complexes in each lead. This generated 2 sets of values for P wave duration for each lead in a given 12 lead ECG. When there was more than 5 ms difference between the values from both the observers, measurements were repeated by both observers on that given lead and compared. A mean of the 2 values were obtained for each lead and based on these values, P-max and P-min were calculated as the maximum P wave duration and minimum P wave duration respectively in a given 12 lead ECG. P

wave dispersion (PWD) was calculated as the difference between P-max and P-min. These values were reported for all the study participants at baseline and following n-3 PUFA supplementation. At study completion and un-blinding, the PWD values between the two groups were compared to evaluate the effect of n-3 PUFA on this index of atrial arrhythmogenesis.

2) Heart rate variability (HRV): Day 3 of each patient's ECG recording was analysed for 24-hour time and frequency domain HRV parameters using a commercial software package designed for this purpose (Pathfinder HRV Tools software package). Additional single hour frequency domain measures were taken from the day 3 data for one hour prior to AF onset, or between 12pm-1pm if no AF had occurred. Episodes of ECG of non-sinus rhythm >10 seconds were marked and excluded from system calculations of HRV measures. The time and frequency domain parameters collected are summarised in table-7, and are as recommended by ESC and NASPE (Heart Rate Variability Task Force 1996)[127]. When calculating the HRV parameters only normal-to-normal (NN) intervals were used. Thus, both ectopic coupling intervals and post-ectopic pauses were excluded from the measurements.

The time domain measures determine the variations in successive heart beats allowing temporal analysis. They can be thought of as follows: SDNNi estimates the overall HRV and mirrors circadian rhythm; SDANN estimates the long term components of HRV; RMSSD estimates the short term components of HRV and higher values indicate vagal activity.

Power spectral analysis provides information of how power (i.e. variance) distributes as a function of frequency. The frequency domain measures are less well

defined but may be thought of in the following terms; nHF represents vagal activity; nLF may mirror sympathetic modulation and LF/HF reflects sympathovagal balance.

Table-7: Description of HRV measurements

HRV Measurement	Description
Time Domain Measures	
SDNNi (ms)	Standard deviation of the NN intervals in every 5 mins, a single 24hr value is reported
SDANN (ms)	Standard deviation of average NN interval for every hour, a 24hr value is reported
RMSSD (ms)	Square-root of the mean of the sum of all squares of differences
Frequency Domain Measures	
nLF	Low frequency power (0.04-0.15Hz) in normalised units
nHF	High frequency power (0.15-0.40Hz) in normalised units
LF/HF	Ratio of LF/HF

2.1.12: Estimation of serum and tissue levels of n-3 PUFA

All serum and tissue samples were stored at -80°C. Samples for fatty acid analysis were later transported on dry ice to the University of Southampton, where they were maintained at-80°C until analysis. Serum CRP concentrations were measured by a previously validated technique of particle-enhanced immuno-turbidimetric assay (Modular P600, Roche Diagnostics, Mannheim, Germany) using ruthenium electro-chemiluminescence to obtain a signal.[128,129] The fatty acid compositions of serum phosphatidyl-choline (PC), the major circulating phospholipid, and of atrial tissue PC and phosphatidyl-ethanolamine

(PE) were determined using gas chromatography. In brief, total lipid was extracted from serum or homogenized atrial tissue using chloroform/methanol (2:1 vol/vol). PC and for atrial tissue PE were isolated from the total lipid extract using aminopropylsilica solid-phase extraction columns; PC was eluted with chloroform/methanol (3:2 vol/vol) and PE with methanol. Fatty acid methyl esters were prepared from the isolated PC and PE fractions by incubation with methanol containing 2% sulfuric acid for 2 hours at 50°C.

Fatty acid methyl esters were isolated by solvent extraction, dried, and separated in a gas chromatograph (model 6890; Hewlett-Packard, Avondale, PA) fitted with a 30m x 0.32 mm BPX70 capillary column with a film thickness of 0.25 mm. Helium at 1.0 ml/min was used as the carrier gas and the split-splitless injector was used with a split-splitless ratio of 20:1. Injector and detector temperatures were 275°C. The column oven temperature remained at 170°C for 12 min after sample injection and was programmed to then increase to 210 °C in increments of 5°C/min and then remain at 210°C for 15 min. The separation was recorded with HP GC CHEM STATION software (Hewlett Packard). Fatty acid methyl esters were identified by comparison with standards run previously and data are expressed as percentage of total fatty acids present in the PC or PE fraction.

2.1.13: Statistical analysis of clinical data

All data were analyzed by a qualified statistician. All analyses were done on an intention-to-treat basis. Comparisons between groups were made using χ^2, Student *t* test, and Mann–Whitney *U* tests as appropriate. Differences in AF-free survival were assessed using Kaplan–Meier analysis. Cox proportional hazards regression analysis was performed to assess the confounding effect of baseline patient characteristics. Variable selection was done using a stepwise selection process. All analysis was carried out using SPSS Version 15.0. In all cases, a value of $P<0.05$ was taken to indicate statistical

significance. All continuous variables are reported as median with interquartile ranges (25th to 75th percentiles).

2.2: Quantitative PCR

The high sensitivity of Quantitaive PCR (qPCR) makes it an ideal technique to detect expression of ion channels, transporters and calcium handling proteins as the mRNA expression of these channels in cardiomyocyte is considered to be relatively low (compared to skeletal muscle, CNS tissue etc). In addition, this technique can be used to detect large number of target mRNAs quantitatively in small tissue samples. Most reagents for qPCR are available as commercial kits. The main principle of PCR relies on exponentially amplifying cDNA, obtained from mRNA by a reverse transcription reaction, using a polymerase chain reaction. Recent technological improvements and the use of fluorescent systems enable amplification to be recorded throughout the time course of the PCR. This allows for small differences in the abundance of the initial gene transcript to be accurately estimated. Thus all qPCR involves the use of fluorescence to detect the threshold cycle (Ct) during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplified curve. SYBR Green is a dye that intercalates with double-stranded DNA. This intercalation causes the SYBR to fluoresce. The qPCR machine detects the fluorescence and software calculates Ct values from the intensity of the fluorescence. Steps followed in this the qPCR procedure were as follows

(1)Total RNA isolation from the right atrial appendage tissue

(2) Determination of the quantity and quality of the RNA isolated

(3) cDNA synthesis- Reverse Transcription

(4) qPCR with primers specific for the target mRNA

(5) Calculation of the relative abundance of the target mRNA.

2.2.1: Total RNA isolation from the right atrial appendage tissue

The sample of frozen right atrial appendage tissue was cut in 10-20 µm sections on a cryostat and used for total RNA isolation using a RNeasy Micro Kit (Qiagen). 155 µl of lysis buffer (miRVana) was added to the tissue sections and immediately homogenized with a polytron at full speed for 2 minutes. This ensured quick and efficient denaturation of RNases. RNase free water was then added to the mixture (295µl) followed by addition of proteinase K (5µl) (Quiagen). The mixture was incubated in a water bath at 55°C for 10 minutes until a clear solution was visible. This allowed a vast majority of proteins to be digested. The sample was then centrifuged at 13000 rpm for 5 minutes and the supernatant transferred to a 1.5ml tube. 150µl of 100% ethanol was applied and the samples were spun through qiagen columns at 10000 rpm and the flow through was removed. DNase stock solution was made up containing DNase and RDD. 350 µl of RW1 buffer was applied to the spin columns and spun through at 10000 rpm for 15s and the flow through was removed. 80µl of RDD/DNase mix was carefully added to the middle of the silica matrix of the column for 20 min. This allowed for the digestion of genomic DNA. 350µl of RW1 buffer was applied to the spin columns and spun through at 10000 rpm for 15 s followed by the addition of RPE buffer (500µl) and a 15s spin at 10000 rpm. A further spin for 2 min with 80% ethanol (500µl) was carried out and then another spin at 13000 rpm for 5 min to ensure the columns were dry. The RNA was then eluted in 14µl of DEPC- treated Millipore H_2O.

2.2.2: Determination of the quantity and quality of the RNA isolated

The major factor that determines the reliability of the outcome in qPCR is the quality of the RNA that is extracted. If RNA is partially degraded then quantification of cDNA synthesized from such RNA extract will lead to misleading results as reported by Bustin et al [130]. Hence, the quality of extracted total RNA was assessed using Agilent NanoLabChips run on the Agilent 2100 Bioanalyser. In this method, each nanolab chip is pre-poaded with a gel-dye mix to fill the microchannels. 1µl of each RNA sample is loaded into one of the 12 wells in the NanoLabChips along with a standard ladder (RNA600 ladder). The dye contained within the microchannels intercalates with the RNA and an electropherogram plot of the RNA is obtained. This allows the 18S:28S ratio to be obtained and provides a numerical value ranging from 1-10 with 1 being most degraded. We accepted values over 7 as being indicative of good quality RNA in this study.

The concentration of RNA was quantified by using a NanoDrop 1000 spectrophotometer (Thermo Scientific) which produces accurate readings of RNA concentrations from 1µl of sample. This enables equal amounts of RNA to be used as templates for the reverse transcription reaction which generates the cDNA.

2.2.3: cDNA synthesis- Reverse Transcription

530ng of total RNA (based on the NanoDrop quantification) was used in the reverse transcription reaction to generate cDNA. This was done using the Superscript III First Stand cDNA Synthesis System (Invitrogen) using random hexamer primers as per the manufacturer advised protocol. As the efficiency of each RT reaction can vary, all samples were reverse transcribed at the same time using the same master mix. The cDNA samples thus obtained were diluted in 1 in 35 with molecular biology grade H2O and stored at -20°C.

2.2.4: qPCR with primers specific for the target mRNA

Real time qPCR (RT-qPCR) measures fluorescence emitted during the reaction as an indicator of the DNA amplified during each cycle (i.e. in real time). The fluorescence is generated by a reporter dye which in our case was *Power* SYBR-green (Applied Biosystems). When *Power* SYBR-green is unbound in the solution, it does not emit any fluorescence but when it binds with the minor groove of the double stranded DNA it emits strong fluorescent signal. Thus, the intensity of this fluorescence is directly proportional to the amount of double stranded DNA present. Recording the amount of fluorescence emitted during each cycle enables the monitoring the PCR reaction during the late exponential phase where the first detectable increase in the amount of the target template occurs. Thus, the greater the starting copy of the cDNA target, the sooner a significant increase in fluorescence occurs.

Primers: As we were interested in evaluating the influence of n-3 PUFA on the expression of relevant ion channels, transporters and calcium handling proteins, we predominantly used commercially available kits (QuantiTect Primer assays) which have been tested in human tissue in our laboratory. QuantiTect primer assays are pre-designed and tested primer sets that work with *power* SYBR-green technology and produce reliable and accurate results. They eliminate the requirement of developing and optimizing primer pairs. However, there were few targets for which we had in-house primers designed and tested in human cardiac tissue in previous studies which we used in this study. The primers used in this study are listed in table-8.

Table-8: Primer assays used for qPCR.

A) Qiagen QuantiTect primers

	Target mRNA	Qiagen QuantiTect primer assay number
Voltage-gated Na^+ channels:	$Na_v1.5$	QT00091812
	$Na_v\beta1$	QT00066080
Voltage-gated Ca^+ channels:	$Ca_v1.2$	QT00053480
	$Ca_v1.3$	QT00076657
Voltage-gated K^+ channels:	$K_v1.5$	QT01003177
	$K_{ir}2.1$	QT00001022
	$K_{ir}3.1$	QT00030380
	$K_{ir}3.4$	QT00070406
	$K_{ir}6.2$	QT00068607
Calcium handling proteins:		
	NCX1	QT00075376
	RYR2	QT00018368
	Phospholamban	QT01160761
	SERCA2a	QT00077231
Adrenergic receptors:		
	β1	QT00204309
	β2	QT00200011
Muscarinic receptors:		
	M2	QT00092134

B) Custom-designed primers

Target mRNA	Accession number	Primer sequence 5´-3´
28S	M11167	Forward: gttgttgccatggtaatcctgctcagtacg
		Reverse: ctgacttagaggcgttcagtcataatccc
K_v4.3	NM_004980	Forward: tggccttctacggcatcct
		Reverse: gctcggcgttctccctct
Cx40	NM_005266	Forward: gcctcccaacaaaacacagac
		Reverse: cttctggccataacgaacctg
Cx43	NM_000165	Forward: 5'-3' tgaagagcatggtaaggtgaaa
		Reverse: 5'-3' agcaagaaggccacctcaaa

PCR Protocol: 10 µl reactions were set-up consisting of 2x *Power* SYBR-green universal PCR master mix (Applied Biosystems), 1µl of cDNA template and 10X Qiagen primer assay. The following PCR protocol was used: (i) AmpErase uracil-N-glycosylate activation- 2 mins at 50°C (ii) hot start, 10 mins at 95°C (iii) 40 cycles of denaturation-15 s at 95 °C followed by annealing/extension- 1min at 60°C (iv) dissociation curve- 15s at 95°C, 15s at 60°C and 15s at 95°C. The PCR reaction was carried out in a MicroAmp optical 96-well reaction plate (Applied Biosystems) sealed with an optical cover. Each template was tested as a triplicate in the same plate.

qPCR quantification: To correct for any variation of RNA input into the Rt reaction, the relative abundance for each transcript was normalized to the relative abundance for the housekeeper gene. The housekeeper used in this study was 28s subunit and the following equation was used to calculate the relative abundance of cDNA in each sample:

$R_y = (Eff_y)^{\Delta Cty} / (Eff_{hkg})^{\Delta Cthkg}$

Where y is the transcript, Ct is the cycle threshold value, R the relative abundance, hkg is the house keeper gene, Eff is the average efficiency of the PCR (efficiency of PCR is a measure of number of DNA fragments that are copied in a single cycle). The efficiency of the PCR reaction is calculated from the fluorescence at the end of each elongation step using the following equation:

$$\mathbf{Eff = \sqrt{}F_n/F_{-2n}}$$

Where F is the fluorescence measured and n is the cycle number.

2.3: Immnuohistochemistry

Immunohistochemistry is a technique used to detect and localize specific antigens in tissue sections with the use of antibodies. Primary antibodies react with specific antigens and form an antigen-antibody complex. Secondary antibodies that are raised against the primary antibodies and conjugated to fluorochromes are used to detect the primary antibodies. It is also possible to quantify the fluorescent signal produced by these fluorochromes and obtain a semi-quantitative estimate of the abundance of the antigen being detected.

2.3.1: Antibodies used in immunohistochemistry

There are 2 types of anti-bodies- Monoclonal and polyclonal. Monoclonal antibodies are produced in a laboratory by a hybridoma or an antibody-producing cell source for specific antigen. Polyclonal antibodies are produced by injecting laboratory animals with the protein and then collecting serum from these animals. This serum is then purified to obtain antibodies for the specific antigen injected into the animal. Consequently, polyclonal antibodies react against many regions of the molecule thus giving a stronger signal while monoclonal antibodies are specific and may have a weaker signal. Thus, polyclonal antibodies are very sensitive to detect small quantities of antigen while monoclonal antibodies are very specific.

The antibodies used in this study are listed in table-9. The specificity and reliability of most of these anti-bodies have been tested in our laboratory during previous studies using human tissue [131].

Table-9: Primary antibodies used in Immunohistochemistry (+/-WB)

Target protein	Host species	Company	Catalogue number
PLB-A1	Ms IgG	Badrilla	A010-14
NCX1	Ms IgM	Affinity BioReagants	MA3-926
RyR2	Ms IgG	Affinity BioReagants	MA3-916
SERCA2a	Ms IgG	Affinity BioReagants	MA3-910
CAV-1	Rabbit IgG	Sigma	C-3237

2.3.2: The immunohistochemistry protocol

Immnuofluorescence experiments were carried out on 20μm sections cut from frozen samples of human right atrial appendage tissue obtained per-operatively from patients. The sections were cut using a cryotome and collected on Superfrost Plus glass slides. The sections were fixed in 10% neutral buffered formalin (Sigma) and washed three times with phosphate buffered saline (PBS). Subsequently, they were permeabilised by incubating with PBS containing 0.1% Triton X-100 for 30 mins and ten washed in PBS followed by blocking with 1% bovine serum albumin (BSA) in PBS for 1 hour. After washing 3 times, the sections were incubated with the primary antibodies overnight at room temperature. The primary antibodies were diluted in 1% BSA in PBS and used at a dilution of 1:50. The following day, the slides were washed three times in PBS and incubated with appropriate secondary antibody (donkey anti-rabbit IgG conjugated to CY3 and donkey anti-mouse IgG conjugated to cy3) diluted in PBS in a 1 in 100 dilution for 2 hours. Subsequently, slides were washed three times in PBS and sections were mounted with Vectashield (Vector Labs) and sealed with cover-slips and nail varnish. Slides were stored at 4°C and in the dark for subsequent viewing. Fluorescent signal was detected using a confocal microscope (Zeiss LSM5, Carl Zeiss Microscopy) with Pascal software (Zeiss Microscopy). The excitation wavelength used was 550 nm for Cy3. Signal intensity measurements were then made using Volocity software (Improvision) after background correction.

2.4: Western blotting

The Western blot or protein immunoblot is a technique used to detect specific proteins in the given sample of tissue homogenate with the use of gel electrophoresis. The most common type of gel electrophoresis uses polyacrylamide gels (PAGE) and buffers loaded

with sodium dodecyl sulfate (SDS). Initially the protein homogenate is treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) allowing separation of proteins by their molecular weight. SDS-PAGE (SDS polyacrylamide gel electrophoresis) then maintains polypeptides in a denatured state and the proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size measured in kilodaltons (kDa). The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. Lower concentrations of acrylamide lead to better resolution of higher molecular weight proteins. In our study, as we estimated proteins of variable densities, we used commercially available gels with strengths varying from 4% to 10% and frequently a graded gel with multiple strengths along the length of the electrophoretic column to enable adequate separation and accurate identification. A typical Western blot reaction consists of the following steps:

(i) Protein extraction

(ii) Protein quantification

(iii) Sample preparation

(iv) Gel electrophoresis

(v) Transfer of gel to nitrocellulose membrane

(vi) Blocking the membrane (to prevent non-specific binding)

(vii) Treatment with primary antibody

(viii) Treatment with secondary antibody

(ix) Detection and quantification of antigen

(x) Stripping the membrane off the antigen-antibody complex

(xi) Re-probing with a housekeeper gene.

2.4.1: Protein extraction

Frozen human atrial tissue samples were sliced into small cubes using a surgical scalpel and transferred into a 15ml falcon tube and 3mls of Radio-Immuno Precipitation Assay (RIPA) buffer and several protease inhibitors (constituents listed in table-10) was added. The mixture was left in ice and a homogenizer (IKA Ultra-turrax T25) was cleaned with ethanol, Milli-Q water and a final wash with RIPA buffer for approximately 10 sec each. The tissue was then homogenized by inserting the homogenizer into the falcon tube while being kept in ice at a speed of 24000 rpm for 3 x 5 sec. The probe was allowed to cool between each burst of homogenization. This was continued until the tissue was fully homogenized. Between each sample, any residual tissue pieces on the homogenizer were removed with a forceps and the homogenizer was thoroughly cleaned as mentioned above.

Each sample homogenate was centrifuged at 1000 x g at 2°C for 10 minutes to remove un-homogenized tissue bits and residues. The supernatant was then transferred into clean falcon tubes and this process repeated following which the final supernatant was transferred into clean Eppendorf tubes, aliquoted and stored at -80°C.

Table-10: RIPA buffer and protease inhibitors used during extraction of protein

Substance	**Concentration**
Phosphate-buffered Saline (PBS)	1xPBS from a 10x stock solution
Igepal CA360 (non-ionic detergent)	1%
Sodium deoxycholate	0.5%
Sodium dodecyl sulphate (SDS)	0.1%
Protease Inhibitors	
PhenylMethaneSulphonylFluoride (PMSF)	0.1mg/ml
Sodium orthovanadate	1mM
Aprotinin	1µg/ml
Leupeptin	1µg/ml

2.4.2: Protein quantification

One aliquot from each sample was defrozen by transferring it into a container with ice and the protein concentration in that sample was quantified using the Bio-Rad Protein Assay kit. This is a colorimetric assay for protein and is based on the Lowry assay method. The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. The total protein concentration is exhibited by a colour change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. Once the reaction has taken place (about 15 minutes) the absorbance of each sample was read at a wavelength of 750nm using the BioTek ELx 800 Absorbance Microplate Reader and BioTek Gen5TM data analysis software which resulted in an objective measure of the concentration of protein in each sample. The sample with the lowest concentration was used for volume calculations and if any of the samples had such high concentration to

require extremely small volumes, such samples were diluted with the addition of RIPA buffer. Equal quantities (25µl) (not concentrations) of each sample was mixed to make a "control" sample which acted as an internal control and was loaded in one well in experiments.

2.4.3: Sample preparation

Equal concentrations (50µg) of sample protein mixtures were mixed with 1x laemmli sample buffer from a 4x stock solution (table-11) and 5x β-mercaptoethanol and made into a final volume of 50µl with RIPA buffer. The bromophenol blue contained within the laemmli sample buffer is used as a tracking dye to enable visualization of the protein as it runs on the electropheresis gel. The resultant mixtures were then boiled for 5 minutes to reduce the disulphide bonds and destabilize the folded structure of the protein.

Table-11: Constituents of Laemmli's sample buffer

Substance	Concentration
SDS	0.07M
Glycerol	0.018M
4 x Upper gel buffer	50%
Bromophenol blue	0.01M
Milli Q H_2O	Adjust to final volume required

2.4.4: Gel electrophoresis

As I had difficulty in casting very thin gels that were required to separate heavy proteins, I had to resort to commercially available pre-cast gels with strengths ranging from 5%, 7.5% and some graded strength 4-20% (Bio-Rad Ready gel Tris HCL gel). These were

precast polyacrylamide gel containing 10-wells of 30 µl each. The Bio-Rad Mini-Protean II Electrophoresis Cell was used for running all the gels. The gels were carefully removed from the packaging and assembled in the electrode block and 1x running buffer (see table-12) was poured into the chamber and the tank. The gels were then loaded with 20µl of the protein sample prepared. 5µl of Bio-Rad kaleidoscope pre-stained marker of known molecular weight was also added to each gel. The gels were then run at 25V at room temperature for 1 hour or until the dye front had reached the bottom of the gel (or overnight at 4°C for RYR, individual gel strengths used and running conditions are detailed in table 13). Each electrophoresis reaction was run with one well containing a Bio Rad marker and one well containing "control" sample. All experiments were done in triplicate to minimize error.

Table-12: Constituents of running buffer

Substance	**Concentration**
Tris Base	25mM
Glycine	192mM
SDS	3.5mM
Milli Q H_2O	Adjust to final volume required
HCL	pH to 8.3

Table-13: Gel concentrations and running conditions for various proteins of interest

Protein of Interest	Molecular Weight [133-136]	Type of Gel (% separating gel)	Protein Concentration	Running time and rate of transfer
NCX	82KDa	7.5% Tris-Hcl	50µg	25volts constant volts for 1 hour
SERCA2A	120KDa	4-20% Tris-Hcl gradient gel	50µg	25volts constant volts for 1 hour
PLB	22KDa (Dimer-11KDa Monomer 5.5KDa)	15% Tris-HCL	50µg	25volts constant volts for 30 minutes to1 hour
RYR	550 KDa	4-20% Tris-Hcl gradient gel	50µg	25 volts, constant volts, overnight.

2.4.5: Transfer of gel to nitrocellulose membrane

The protein thus separated in an agarose gel has to be transferred to a membrane to enable it to be detected. The commonly used membranes are nitrocellulose and poly vinylidene fluoride (PVDF). In this study, I exclusively used nitrocellulose membrane. The nitrocellulose membrane was chosen for its superior non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes were also cheaper than PVDF membranes. The membrane and some sponge pads were equilibrated with 1x transfer buffer containing methanol (see table-14). Once the gel has completed the electrophoretic reaction, a gel sandwich is made which consisted of two sponge pads in the cathode core over which one piece of filter

paper was laid and the gel was placed on top of this. The nitrocellulose membrane was then placed over the gel and a further filter paper and a sponge were applied on top to complete the sandwich. The blot module was closed and placed in the guide rails on the lower buffer chamber with a gel tension wedge so that the blot module was locked in place. The blot module was then filled with deionised water (milli Q). This serves to dissipate the heat produced during the transfer run. The gels were then transferred at constant voltage for one hour. This method of transferring the proteins is called electroblotting and uses electric current to pull proteins from the gel into the nitrocellulose membrane. The protein move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. After transferring, the membranes were washed briefly in 1x Tris Buffered Saline with 1% Tween^R 20 (TBS-T) (see table-15) to remove any gel debris.

Table-14: Constituents of transfer buffer

Substances	**Concentrations**
Tris Base	25mM
Glycine	192mM
Milli Q H_2O	Adjust to final volume required
NaOH	pH 8.3

Table-15: Constituents of TBS-T

Substance	Concentration
Tris Base	20mM
NaCl	150mM
Tween®20	1%
Milli Q H_2O	Adjust to final volume required
HCL	pH to 7.6

2.4.6: Blocking the membrane (to prevent non-specific binding)

Prior to probing the nitrocellulose membrane with an antibody against the target antigen, the membrane has to be blocked to prevent non-specific binding. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically 3-5% Bovine serum albumin (BSA) or non-fat dry milk (5%) in Tris-Buffered Saline (TBS), with a detergent such as Tween 20 or Triton X-100. In this study, I used 5% milk as a blocking agent. This ensures that the milk protein attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

2.4.7: Treatment with primary antibody

After blocking, the membrane was exposed to primary antibodies against the target antigen (commercially available antibodies generated in a host species or immune cell culture by exposure to the protein of interest was used (see table-9 for details of the primary antibodies used). A dilute solution of primary antibody (5 micrograms/ml), made

by mixing small quantity of antibody with Tris Buffered Saline (TBS) was added to the membrane and the membrane was incubated under gentle agitation. The antibody solution and the membrane were incubated together for variable periods depending on the ease of transfer of the given target protein and this varied between 1-hour to overnight incubation. Shorter incubation was done at room temperature while longer incubation was done at 4°C. Generally warmer temperatures are associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

2.4.8: Treatment with secondary antibody

After treatment with primary antibody, the nitrocellulose membrane was thoroughly rinsed with TBS-T for 3-5 times to remove any unbound primary antibody. Subsequently, the membrane was exposed to another antibody, directed at a species-specific portion of the primary antibody. For example, an anti-mouse secondary will bind to almost any mouse-sourced primary antibody and will provide consistent results for detection of the binding by the primary anti-body. This is known as a secondary antibody. The “secondary” antibody was conjugated to a horseradish peroxidase (HRP) enzyme which acts as a reporter enzyme. Horseradish peroxidase-linked secondary was particularly used to cleave the chemi-luminescent agent, and the reaction product produces luminescence in proportion to the amount of protein.

2.4.9: Detection and quantification of antigen

The pierce Super Signal West Pico Chemi-luminescent substrate, consisting of a stable peroxide buffer and luminol/enhancer solution was used to detect the target proteins. The membrane was incubated with 1ml each of the 2 solutions contained in this commercial kit for 5 minutes and the target protein was visualized directly in a chemiluminescence-

compatible digital imaging system. The conjugated HRP enzyme catalyses the degradation of the chemiluminescent substrate resulting in signal generation.

2.4.10: Stripping and re-probing with house keeper gene

The common confounder in quantifying the protein by this technique is error with loading resulting in variable quantity of proteins being loaded. This can be assessed and if any errors found corrected by stripping the membrane and re-probing with a house keeper gene. The housekeeper gene is a gene which remains constant and the luminescence for all the wells should be equal when probed for the house keeper gene. If there were any disparity, the value of the protein can be normalized to the housekeeper gene which corrects for loading error. In this study, a commercially available stripping agent, Pierce Stripping Buffer was used to strip the antibody from the membrane and the membrane was reprobed with GAPDH as a house keeping gene. All results were expressed after normalizing to GAPDH and to the internal control sample.

2.5: Cell isolation from human right atrial appendage

I attempted to isolate single atrial myocytes from human atrial appendage tissue obtained during CABG surgery. Unfortunately, despite trying various protocols and modification of an apparently successful protocol, I was unsuccessful in consistently isolating good quality cells that could be used to perform cellular electrophysiological experiments. Hence, after trying for over a year, I abandoned this part of the study and decided to obtain similar data from animals (4 week old, male Wistar rats), fed a 5% fish oil diet (which would mimic the dose of human supplementation) for a period of 4 weeks. The protocol used in the attempt to isolate human atrial myocytes is a modification of the methods used by Dobrev D et al[132] and is detailed below.

A portion of the atrial appendage tissue that was removed from study participants was immersed in a cold transport solution (see table-16) immediately upon removal from the patient. This was washed then free of any blood and epicardial fat and sliced into small pieces using a sharp surgical scalpel within few minutes of removal from the patient (in the cardiac surgical theatre annex) and re-suspended in fresh cold transport solution. This tissue was then taken to a laboratory in the hospital within 10 minutes, where the tissue was transferred into a tissue dissociation vessel containing calcium free oxygen-saturated isolation solution (see table-17) which was maintained at 35°C and continuously oxygenated for 20 minutes.

Subsequent cell isolation was attempted by an enzymatic digestion technique. In brief, the following enzymes - collagenase type V (350 I.U. ml^{-1}, Sigma) and protease type XXIV (4 I.U. ml^{-1}, Sigma) were added to the calcium free isolation solution in the vessel containing the tissue. This vessel was maintained at 35°C and continuously oxygenated. The vessel was also continuously rocked in a heated bath to improve tissue dispersion. This step lasted for 15 minutes. After this step was completed, the samples solution was removed and replaced with fresh isolation solution containing only collagenase type V (350 I.U. ml^{-1}) for 30 minutes in similar conditions.

The solution was then replaced by a new enzymatic solution where the collagenase concentration was reduced to 175 I.U. ml^{-1}. During this final step, the supernatant was replaced by fresh collagenase solution every 15 min and the dissociation was followed by microscopic examination of the collected medium.

Once cells were identified, the collected solution was centrifuged at 1000rpm for 5 mins and the supernatant was removed and replaced by Calcium free Tyrode's solution. The Calcium concentration was raised stepwise (50, 100, 200, 500 μM). The isolated cells

were kept at room temperature in Tyrode's solution (table-18)and transported to the university laboratory for use the same day.

The cells, which appeared to be healthy on initial microscopy, were calcium intolerant and got readily damaged upon exposure to calcium. I tried to modify this protocol with varying the concentration of the enzymes used for digestion and attempting to improve calcium tolerance by introducing the tissue continuously to a small concentration of calcium throughout the isolation process. None of these manoeuvres were successful in yielding good quality cells.

Table-16: Constituents of transport solution

Substance	**Concentration (mmol)**
NaCl	103
Kcl	5.4
CaCl2	1.8
Lactic acid	286
NaOH	To adjust pH to 7.0

Table-17: Constituents of isolation solution

Substance	Concentration (mmol)
NaCl	120
Kcl	10
KH2PO4	1.2
MgCl2	1.2
Glucose	10
HEPES	10
Taurine	20
NaOH	To adjust pH to 7.2

Table-18: Constituents of Normal Tyrode's solution

Substance	Concentration (mmol)
NaCl	134
KCl	4
CaCl2	1
MgCl2	1.2
Glucose	11.1
Probenecid	2
NaOH/HCl	To adjust pH to 7.2

2.5.1: Further attempts at human atrial cell isolation

As I was unable to isolate good quality cells from human right atrium, I halted the recruitment process for the trial with a view to avoid loss of samples from trial patients. I then applied for a further ethical permission to perform trial isolation from patients who were not enrolled in the study but would undergo routine CABG surgery as right atrial appendage tissue was routinely excised during CABG surgery to connect the patients' heart to the extra-corporeal circulation. Such ethical permission was obtained from the local (South Manchester) ethics committee. I then used this tissue to try and optimize the isolation process with guidance from the researchers in Dresden, Germany (Dr. Dobrev and Dr. Ravens) who have significant experience in performing experiments on single cardiomyocytes isolated from right atrial appendage tissues. I also visited the lab of Dr.Workman and Dr. Rankin at University of Glasgow, UK to observe the procedure of single cell isolation from human atrial appendage.

The consistent finding from the cells isolated in both these experienced centres was that the cells were variably depolarised with trans-membrane potentials which were higher than usual (around -20 to -40). This was considered to be due to loss of membrane bound ion channels during the isolation procedure. I also realised that these centres used these cells for experimentation after bringing the trans-membrane potential to physiological values by passing a depolarising current. Whilst such a manoeuvre may be acceptable to evaluate the effect of a compound acutely applied (by adding to the perfusate), we were sceptic about the use of such a technique and in any case, such a method would not allow us to compare the effects of a supplemented, incorporated substance such as the n-3 PUFA in diet. Hence after trying for nearly a year, I decided not to pursue this arm of the study and to replace it with animal experiments.

2.6: Animal experiments-methods

4 week old, male Wistar rats were sourced from Charles River Laboratories (UK) and housed in the University of Manchester, biological services facility. Animlas were randomly assigned to received feeds supplemented with 5% n-3 PUFA or a standard chow (control) for a 4 week period. The researcher was blinded to the feed allocation. On the day of the experiment, an animal from one of the 2 groups was transported to a dedicated cell preparation room minutes prior to use. The rat was humanely sacrificed via stunning followed by cervical dislocation in accordance with the guidelines set by the Home Office Animal (Scientific Procedures) act 1986. The whole heart were swiftly removed via a thoracotomy and stored in isolation solution (table-19) where it was gently massaged to assist blood clearance and to prevent clotting. Subsequently, the heart was cannulated via the aorta and attached to a Langendorff apparatus were tissue digestion was carried out by means of retrograde perfusion with solutions containing proteolytic and fibrinolytic enzymes as detailed below.

Table-19: Constituents of isolation solution

Substance	**Concentration (mmol)**
NaCl	134
KCl	4
NaH_2PO_4	1.2
Glucose	11.1
$MgSO_4$	1.2
HEPES acid	10
NaOH	To adjust pH to 7.34

2.6.1: Atrial myocyte isolation- unsuccessful

I initially attempted to perform the field stimulating experiments using isolated rat atrial myocytes. I attempted to isolate atrial myocytes using a protocol that has previously been used by our group (Walden A, Trafford A). In this technique, the heart was retrogradely perfused (37 °C) at 8 ml-min^{-1} with solution A (details below). Once the heart had cleared of blood and was showing regular contractions solution B was perfused for 4 min. Collagenase (Worthington type 1, 0.8 mg.ml^{-1}) and protease (Sigma type XIV, 0.08 mg.ml^{-1}) were then perfused for 10 min in solution C. After enzymatic digestion the atria were dissected free from the ventricles and gently agitated in solution C containing 1% bovine serum albumin at 37 °C and cells filtered through 200 μm nylon gauze and centrifuged gently at 40 g for 60 s. The supernatant was discarded and the cells re-suspended in solution C and attempted to be used for experimentation. However, despite our best efforts, this isolation technique was not yielding good quality cells that were calcium tolerant, consistently and we had to abandon this step and move on to performing the experiments in isolated rat ventricular myocytes.

Solution A (mmol.L^{-1}): NaCl, 130; KCl, 5.4; $MgCl_2$, 1.4; NaH_2PO4, 0.4; glucose, 10; HEPES, 5; creatinine, 10;taurine, 20; $CaCl_2$, 0.5 (pH 7.34 with NaOH).

Solution B (mmol.L^{-1}): NaCl, 130; KCl, 5.4; $MgCl_2$, 1.4; NaH_2PO4, 0.4; glucose, 10; HEPES, 5; creatinine, 10; taurine, 20; EGTA, 0.1 (pH 7.34 with NaOH) .

Solution C (mmol.L^{-1}): NaCl, 130; KCl, 5.4; $MgCl_2$, 1.4; NaH_2PO4, 0.4; glucose, 10; HEPES, 5; creatinine, 10; taurine, 20; (pH 7.34 with NaOH).

2.6.2: Ventricular myocyte preparation

The Langendorff apparatus consists of a coil into which solutions from measuring cylinders within a water bath (Grant Instruments Ltd, UK) maintained at 37°C, enter via a Miniplus 3 peristalytic pump (Glison Inc, Wisconsin, USA). Surrounding this coil is a water jacket, into which the water bath pumping unit pumps water with the result of maintaining a solution temperature of 37°C. The coil terminates at a bubble trap, after which solutions may enter the cannula and thus the heart, perfusing the tissue via the coronary arteries. Control of solution flow is achieved via a three way tap downstream of the peristalytic pump. Tubing upstream of the tap is kept as short as possible to reduce the lag time of the desired solution reaching the heart. Before and after each solution, the Langendorff apparatus was flushed with ultrapure water from the Milli-Q water purification system (Millipore (UK) Ltd). The procedure for myocyte isolation is as follows. Two solutions were used for cell isolation 1) a calcium free isolation solution as shown in table-19 and 2) a low calcium taurine solution as shown in table-20. Initially, the Langendorff apparartus was primed by filling the tubing, coil and bubble trap with isolation solution. Bubbles were kept to the minimum so as not to exhaust the bubble trap reserves and to reduce the dead space in the system. Following canulation, the heart was perfused with isolation solution in order to flush the coronary arteries and heart chambers off blood. It is essential to flush all the blood as clotting will prevent perfusion and in addition calcium present in the blood can affect the activity of the digestive enzymes used. Following the initial washout phase, the heart was perfused with isolation solution containing collagenase (Worthington Biochemical Corporation, New Jersey, USA) and protease (Type XXIV, Sigma Aldrich) at concentrations of 0.6mg/ml and 0.067mg/ml respectively. The digestive solution was allowed to perfuse the heart for a typical duration of 7-8 minutes after which the heart was flushed with taurine solution which removed all

the residual enzymatic activity. At the end of this digestive process, the heart was removed from the Langendorff apparatus and the ventricles were dissected out. The ventricles were then transferred into a 20ml beaker containing taurine solution where it was gently chopped and the tissue triturated using a truncated Pasteur pipette. The tissue was then allowed to settle and the supernatant poured via a fine mesh (Bio-Design Inc, New York, USA) into a test tube. This first stage served as the final wash after which the tissue was repeatedly processed to extract cells using the same method of finer chopping and trituration in a smaller volume of taurine solution. After each processing, the supernatant solution was filtered through the fine mesh to collect cells and this was repeated 4-5 times so that several suspensions of single cells were obtained.

Table-20: Constituents of taurine solution

Substance	**Concentration (mmol)**
NaCl	115
KCl	4
NaH_2PO_4	1.2
Glucose	11.1
$MgSO_4$	1.2
HEPES acid	10
Taurine	50
NaOH	To adjust pH to 7.34

2.6.2: Myocyte storage and dye loading

After isolation, the cells were re-suspended in taurine solution so as to improve the ratio of live cells to debris. The tubes were then inspected for cell quality under an epifluorescent inverted binocular microscope (Nikon, UK). Rod shaped, clearly striated cells that did not spontaneously oscillate were indicative of good quality cells and test tubes containing large number of such cells were chosen for experimentation. 1 ml of cell suspension was then loaded with Fluo-3AM (Molecular Probes, Invitrogen, UK).

The Fluo 3AM indicator was made up with 20% w/v dimethyl sulfoxide (DMSO)/pluronic F127 to a stock concentration of 1mM. DMSO solubilises the AM ester. Pluronic F127 is a low-toxicity dispersing agent which facilitates cell loading. The cells were loaded with 5µM Fluo-3AM for 10 minutes then diluted in tyrodes solution to prevent further loading. Cells were allowed to stand for at least 30 minutes for de-esterification. This allows time for non-specific esterases to cleave the AM ester, releasing the free acid form which will remain trapped in the cytoplasm. In addition, 2mM Probenecid (Sigma Aldrich, UK) was routinely added to the suspension to prevent Fluo-3 leaving the cell via anionic sarcolemmal channels.

2.6.3: Experimental set-up

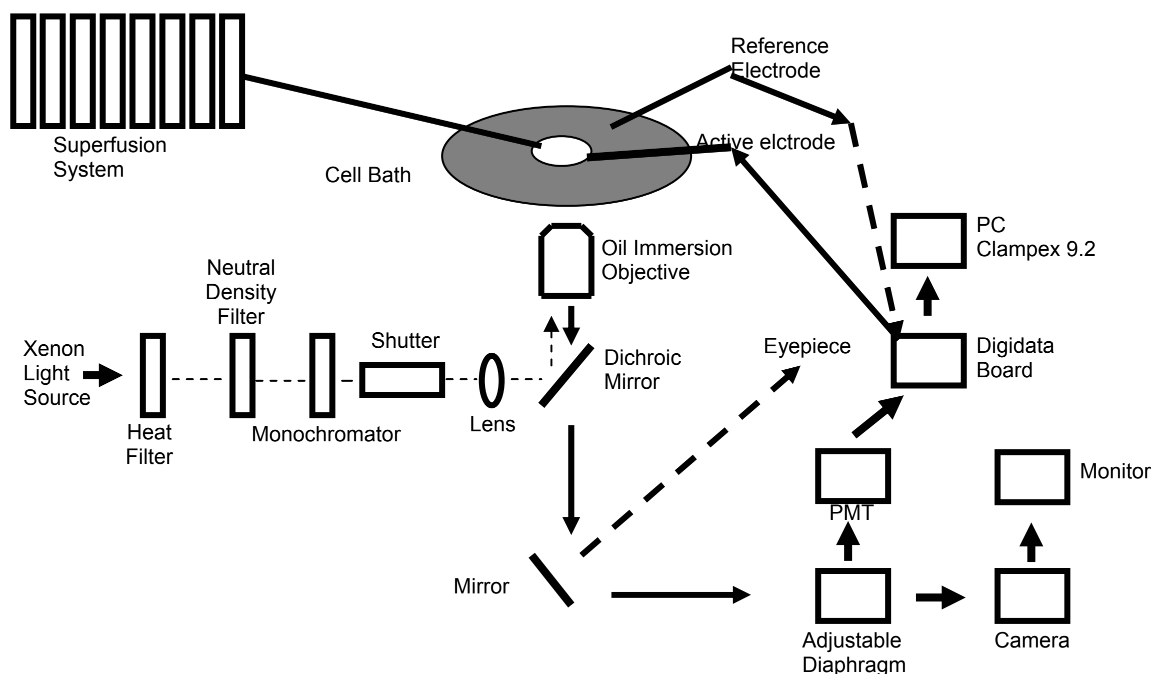
The experimental solutions were contained within eight glass cylinders attached to the perfusion chamber where the cells were situated, via plastic inflow tubes and a solution changer with heated tip. Solenoid valves were in place, to allow the flow from the different cylinders to be controlled. Prior to experimentation, a mounting chamber (bath) was prepared by affixing a cover slip (using silicone grease) over a rectangular aperture in a Perspex disc. This assembly was then secured in place on the stage of the inverted microscope (Olympus IX71, Japan). The bath was then washed with Normal Tyrode's

solution and an aliquot of loaded cells was then added to the bath and allowed to settle down and adhere to the cover slip base of the bath. The solution contained in the well was continuously replenished by gravity driven inflow at a rate of 0.6ml/min from the superfusion system and removed by an outflow tube connected to a suction pump. Approximately 200µl of cell suspension was added to the well each time and allowed to settle for five minutes to ensure the cells attached to the glass cover-slip before the perfusion system was turned on. This allowed the removal of any unattached cells.

2.6.4: Field stimulation

All experiments were carried out in room temperature. Myocytes were field stimulated to contract via Ag/$AgCl_2$ electrodes at a fixed frequency of 0.5Hz. The electrodes were affixed to the chamber with the ends of the wires placed at opposing sides of the well. The electrodes were connected to a constant voltage stimulator Digitimer DS2. The cells were visualised using an Olympus x40 U-Apo 340, NA 1.35 oil immersion objective. Using field stimulation, systolic calcium transients at control condition (Normal Tyrodes) was obtained following which the bath was superfused with a 10mM caffeine containing solution which caused release of calcium from the sarcoplasmic reticulum (SR) as caffeine greatly increases the open probability of ryanodine receptors and causes the SR to empty almost all of the calcium contained within it. The calcium transient thus obtained is a reflection the total SR content of calcium. Subsequently, nickel plus caffeine was added which evoked calcium transients that allowed for measurements of NCX correction factor and fractional contribution of SERCA, NCX and PMCA to calcium removal.

Figure-2.2: Experimental set-up for field stimulation (to measure fluorescence)



2.6.5: Measurement of calcium concentration and cellular electrophysiology.

Autofluorescence: Cells contain molecules which are known to fluoresce when excited with UV radiation, in the absence of a fluorescent indicator. The majority of cell auto-fluorescence originates from mitochondria and lysosomes with the most important endogenous fluorophore being nicatinamid adenine dinucleotide phosphate (NADPH) coenzymes. This kind of background fluorescence is small compared to that produced by the fluorescent indicator.

Calcium quantification derived from fluorescence: In some cases the cells were not accurately calibrated to calcium due to a failure to obtain a maximal fluorescence for fluo-3AM (fmax) measurement at the end of the experiment. Therefore intracellular calcium is expressed as F/F_0, using the following equation:

$$F/F0 = (F - \text{no cell})/(F_0 - \text{no cell})$$

where F is the raw fluorescence measurement, F_0 is fluorescence at diastolic levels and the no cell is the background fluorescence. Background fluorescence is determined by the measurement of fluorescence in the absence of a cell.

2.6.6: Data analysis

All data were acquired to the PC using Clampex version 9.2, Axon Instruments Inc., USA. The files were exported into a visual basic programme (written 'in house') for analysis. All graphs were plotted in Sigmaplot version 8.0 (SPSS Inc., USA).All data are shown as means ± standard error of the mean (SEM). Statistical significance was determined using a Students t-test where appropriate; however data that was not normally distributed was transformed by log10 prior to performing a Students t-test. In some cases data could not be transformed to achieve a normal distribution and a Mann Whitney Rank Sum test was used. A p-value of less than 0.05 was considered significant.

Chapter 3

CHAPTER-3: RESULTS

3.1: Clinical outcome

3.1.1: Demographics and duration of supplementation

Of the 108 patients recruited into the study, 103 patients completed the study. Fifty-one patients were randomly assigned to receive placebo, and 52 received n-3 PUFA. Of the 5 patients who did not complete the study, 2 died awaiting surgery (both were in the placebo arm), 2 had their surgery cancelled, and 1 withdrew consent. There were no clinically relevant differences between the 2 groups with respect to baseline variables such as demographics, co-morbidities, and other likely confounding factors, as shown in table-21.

Table-21: Baseline characteristics of patients in each treatment group

Variables	Placebo (n=51)	n-3 PUFA (n=52)
Male	82% (42)	77% (40)
Age; median (IQR), y	68 (64–73)	64 (58–71)
Body mass index; median (IQR), kg/m^2	27.2 (24.9–30.2)	28.3 (26.1–31.5)
β-blockers	82% (42)	88% (46)
ACE inhibitors/ARB	88% (45)	79% (41)
Calcium antagonists	26% (13)	19% (10)
Statins	98% (50)	98% (51)
Hypertension	29% (15)	35% (18)
Diabetes mellitus	16% (8)	13% (7)

COPD	10% (5)	8% (4)
Renal impairment	8% (4)	6% (3)
Previous myocardial infarction	28% (14)	23% (12)
Echo LV function, low, ≤55%	8% (4)	10% (5)
Echo LA size, dilated, ≥2.3 cm/m^2	6% (3)	4% (2)
Postoperative inotropes	53% (27)	58% (30)
No. of vessels grafted		
1	8% (4)	6% (3)
2	39% (20)	33% (17)
3	49% (25)	56% (29)
4	4% (2)	6% (3)
Bypass time; median (IQR), min	72 (34, 245)	88 (25, 245)
Cross-clamp time; median (IQR), min	50 (20, 130)	61 (19, 126)
Use of Anti-Arrhythmic drugs (Amiodarone)	7	6

IQR: interquartile range; ACE: angiotensin-converting enzyme; ARB: angiotensin-receptor blockers; COPD: chronic obstructive pulmonary disease.

The median duration of therapy before surgery was 17 days (12–20) in the placebo group and 16.5 days (13–21) in the n-3 PUFA group, as shown in table 21.

3.1.2: Drug compliance and levels of n-3 PUFA

All participants who completed the study (103 out of 108 recruited) reported good drug compliance and this was verified by "pill count" of the returned bottles of the trial agent by a clinical pharmacist. In addition, the serum, PBMC and tissue levels of n-3 PUFA among the two groups were significantly different with consistently higher levels in all the three among those who received n-3 PUFA as shown in table-22.

Table-22: Serum, PBMC and cardiac tissue levels of n-3 PUFA

Fatty Acid	Time Point	Placebo	n-3 PUFA	P^*
Serum EPA	Study entry	1.76 (1.49)	1.51 (0.98)	NS
Serum EPA	Surgery	1.53 (0.74)	2.78 (1.13)	<0.001
Serum DHA	Study entry	3.82 (1.38)	3.79 (1.37)	NS
Serum DHA	Surgery	4.19 (1.29)	5.49 (1.02)	<0.001
PBMC EPA	Study entry	0.76 (0.6)	0.61(0.41)	NS
PBMC EPA	Surgery	0.67 (0.39)	1.19 (0.51)	<0.001
PBMC DHA	Study entry	2.53 (0.72)	2.34 (0.70)	NS
PBMC DHA	Surgery	2.60 (0.74)	3.16 (0.57)	<0.001
Tissue PC EPA	Surgery	0.69 (0.30)	1.23 (0.50)	<0.001
Tissue PC DHA	Surgery	3.3 (1.1)	4.5 (1.6)	0.007
Tissue PE EPA	Surgery	1.73 (0.73)	2.7 (1.0)	0.002
Tissue PE DHA	Surgery	11.6 (3.7)	14.3 (3.9)	0.037

Values are mean (SD), percent of total fatty acids. *Student *t* test, unpaired. PC:Phosphatidyl choline, PE: Phosphatidyl ethanolamine.

3.1.3: Primary clinical outcome

The primary outcome of interest, which is any AF of more than 30s duration in the 5 days following coronary artery bypass surgery occurred in was 49.5% (i.e. 51 patients of 103 had at least 1 episode of AF ≥30 seconds). This incidence is higher than most previously reported incidence even though within the range of the reported values. The reason that the overall incidence is high is likely to be due to the robust monitoring for AF rather than a true increase in the study subjects. When individual groups were evaluated, it was noted that postoperative AF had occurred in 43% of the placebo group and 56% of the n-3 PUFA group, with a difference of 13% between the 2 groups (95% confidence interval [CI], −6% to 30%; $\chi 2$ test; P=0.28) (table-23). This is clearly an unexpected result as the patients who were supplemented with n-3 PUFA had **higher** incidence of AF but it is important to note that the difference was not statistically significant and therefore could have occurred by chance. All data that was not normally distributed was analysed using a non-parametric test (Mann- Whitney U test) and are expressed as inter quartile ranges (IQR).

Table-23: Duration of therapy and clinical outcomes

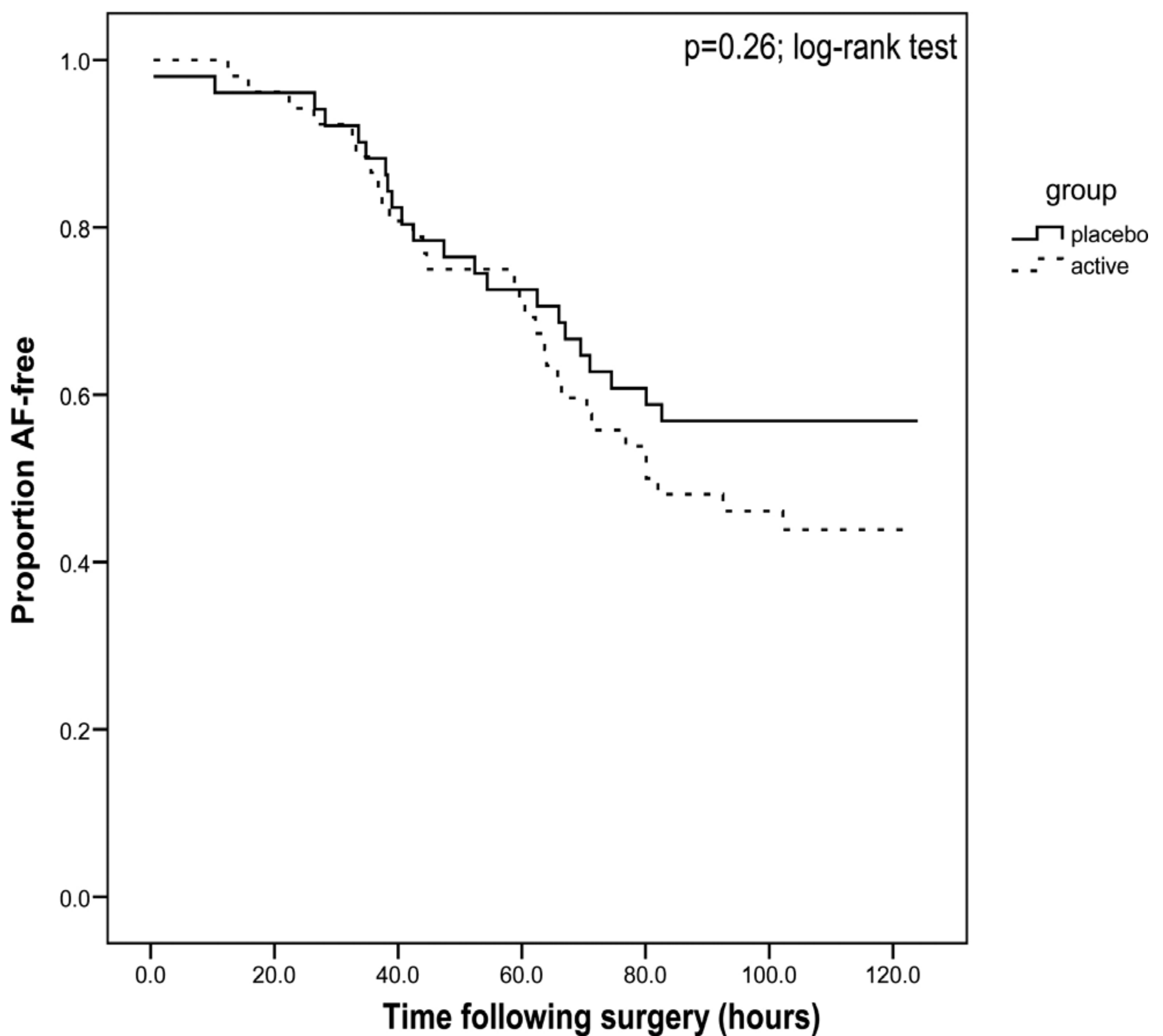
	Placebo (n=51)	**n-3 PUFA (n=52)**	***P***
Any AF ≥ 30sec	43% (22)	56% (29)	0.28*
Clinical AF, % (n)	35% (18)	42% (22)	0.60*
AF burden; median (IQR), % hours	9.6 (4–20)	13.2 (5–30)	0.49†
Hospital stay; median (IQR), d	7 (6–10)	8.5 (6–12)	0.49†
ICU/HDU stay; median (IQR), d	1 (1–2)	1 (1–2)	…
Duration of therapy; median (IQR), d	17 (12–20)	16.5 (13–21)	…

Values are median (IQR) for continuous variables, ICU indicates intensive care unit; HDU, high-dependency unit. *χ^2 test; †Mann–Whitney *U* test.

The Kaplan-Meier actuarial estimates of the occurrence of the primary end point of AF ≥30 seconds after CABG are shown in figure-3.1. This represents the time since the surgery that a given patient was free from any atrial fibrillation. This again depicts the fact that there was no significant difference in the AF-free distribution between the 2 groups (log rank test; P=0.26). The AF free survival time started declining about 20 hours after surgery and reached a plateau at about 80 hours following surgery again indicating that most incidences of AF occurred in day 2 and 3 after surgery. This is in keeping with routine clinical observation that AF following surgery occurs most frequently within the first 3 days following surgery. In our study, maximal incidence of AF occurred between 40 to 80 hours after surgery (59%). There were no new episodes of clinical AF after 5

days of surgery (after the period of continuous monitoring). However, after the period of continuous monitoring, if the patients were still in hospital, they only received a 12 lead ECG each day which might not have accurately quantified AF as patients could have had short bursts of AF when not having the ECG. Specific questioning of patients about symptoms of palpitations did not suggest this to be the case.

Figure 3.1: Kaplan Meier curve for AF free survival in each group



3.1.4: Analyses to assess confounding influence of other variables

As there are several known clinical situations that can pre-dispose individuals to AF we performed a Cox proportional hazards regression analysis to assess the confounding effect of baseline patient characteristics on the relationship between n-3 PUFA therapy and AF. This analysis showed age and number of coronary arteries grafted as the only variables of importance in univariate analysis; multivariate analysis, adjusting for these differences, confirmed that n-3 PUFA therapy had no effect on the incidence of AF, as shown in Table-24.

Table-24: Multi-variate analysis to assess influence of confounding variables

	Hazard Ratio (95% CI)	
	Univariate	Multivariate
Treatment group, PUFA	1.37 (0.79, 2.30)	1.48 (0.84, 2.60); *P*=0.18
Sex, male	0.74 (0.39, 1.41)	
Age, y	1.03 (1.001, 1.06)	1.04 (1.002, 1.07); *P*=0.036
β-blockers	0.79 (0.39, 1.63)	
ACE inhibitors/ARB	0.52 (0.27, 1.02)	
Calcium antagonists	0.95 (0.49, 1.85)	
Statins	0.72 (0.10, 5.18)	
Echo LV function, low	1.32 (0.56, 3.10)	
Echo LA size, dilated	1.09 (0.34, 3.50)	
Postoperative inotropes	0.96 (0.55, 1.66)	
No. of vessels grafted	1.56 (1.03, 2.36)	1.51 (0.99, 2.30); *P*=0.055
Bypass time, 10 min	1.03 (0.97, 1.09)	
Cross-clamp time, 10 min	1.05 (0.95, 1.15)	

ACE: angiotensin-converting enzyme; ARB: angiotensin-receptor blockers.

3.1.5: Analysis of secondary outcomes

i) Clinical incidence of AF

In addition to monitoring for AF using a continous ECG monitor, we also prospectively documented AF as noticed and documented by the clinicians (Anaesthetists, Surgeons, ITU physicians and Nurses) in the patients medical and nursing records. This was pre-defined as one of the secondary end points as this would have relevance to the patient and the treating physicians in that these would have been symptomatic episodes of AF which often is due to a prolonged or troublesome episode needing specific intervention to correct it. We noticed that the overall incidence of clinical (most likely symptomatic or prolonged episodes) AF was 38.8%, with no difference between groups (35% in the placebo group versus 42% in the n-3 PUFA group; χ^2 test;*P*=0.60).

ii) AF burden

This is a measure of the amount of time spent in AF by a given patient during his/her post-operative period. We estimated this as we felt that the highly sensitive technique that we were using to identify AF and the rigid definition (>30s) for an end-point event (internationally accepted definition of an AF episode) might lend itself to a possibility that a given patient might have spent a very small percentage of time in AF but still qualifying as having reached an end point. This again showed that the AF burden was considerable in both groups with an average duration of 9.6 [4 to 20] [% hours] in the placebo group versus 13.2 [5 to 30] [% hours] in the n-3 PUFA group. This difference between the two groups was again not statistically significant (Mann–Whitney *U* test; *P*=0.49). Of the patients who developed AF, most had reverted to sinus rhythm with or with out treatment. 2 patients in the n-3 PUFA group and 1 patient in the placebo group were in AF at the

time of discharge. All the 3 patients were found to be in AF during the 3 week follow-up thus making it persistent AF.

iii) Hospital stay and Intensive Care Unit/High-Dependency Unit stay

One of the major morbidity associated with developing AF in the post operative period is prolonged stay in the high intensity care environment (Intensive/ High dependency care units) with the attendant risk of developing iatrogenic (hospital acquired) illnesses and increased health care costs. Hence we measured this variable to see if there was a difference in this outcome as a result of n-3 PUFA supplementation. In our study we noted that the length of hospital stay was not different between groups, with a median hospital stay of 7 days (6 to 10 days) for the placebo group and 8.5 days (6 to 12 days) for the n-3 PUFA group (Mann–Whitney *U* test; *P*=0.49). Similarly, the length of stay in intensive care/high dependency care was not different with a median ICU/HDU stay of 1 day (1 to 2 days) for the placebo group and 1 day (1 to 2 days) for the n-3 PUFA group.

iv) Concentration of C-reactive protein (CRP) in the serum

CRP is an acute phase protein that is released in the circulation during episodes of active systemic inflammation. Highly reactive CRP is a measure of underlying systemic inflammation and would be expected to be slightly elevated in the post-operative period. As discussed earlier, there is increasing evidence that AF may be an inflammatory disease with many inflammatory conditions pre-disposing patients to AF. n-3 PUFA has been reported to reduce inflammation in acute inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease. Hence in our study we measured HR-CRP in the serum of all patients in samples collected within 24 hours of surgery and on day 3 (48

to 72 hours) after surgery. There were no clinically relevant differences in CRP at either time point between the 2 groups, as shown in table-25.

Table-25: CRP concentration in the treatment groups

Time Point	**Placebo (n=51)**	**n-3 PUFA (n=52)**	***P***
Within 24 h of surgery, mg/L	32 (2–49)	28.5 (15–38)	0.64
Day 3 after surgery, mg/L	188 (79–226)	187.5 (132–229)	0.89

Values are median (IQR) (Mann-Whitney *U* test)

3.1.6: Dietary n-3 PUFA Intake

Dietary intake of n-3 PUFA, particularly of marine source is likely to be a confounder if there was a significant difference between the two groups. This has not been adequately addressed in most studies using n-3 PUFA supplements. We evaluated this administering a food frequency questionnaire which was completed by all the patients who enrolled into the study. Assessment of weekly dietary n-3 PUFA consumption was done by computing the values for each food item and portion consumed over a week and the total value was divided by 7 to obtain average daily n-3 PUFA intake. Such evaluation at baseline did not show a difference between the two groups as shown in table-26.

Table-26: Dietary intake of n-3 PUFA

Treatment group	**Dietary EPA+DHA (mg/day)**	***P***
Placebo	118 (31 to 431)	0.86
n-3 PUFA	131 (24 to 524).	

3.1.7: Postoperative Complications

Post operative complications were evaluated by looking at patient records 4 weeks after discharge and any hospital admission in this period was evaluated for causality of the trial drug (n-3 PUFA). In accordance with the regulations of the Medicines and Health care Regulatory Agency of the UK, all these episodes were reported as adverse events. Three patients were hospitalized (2 in the placebo group and 1 in the active treatment group) in the 4 weeks after discharge with complications that were deemed unrelated to study participation. Two patients (1 in each group) had skin infection over the vein graft site and 1 (placebo group) had non-cardiac chest pain.

3.1.8: P-wave parameters in the two groups

The P-wave parameters analysed between the two groups on standard 12 lead ECG did not show significant differences at baseline. When evaluated for the effect of the intervention (Placebo Vs n-3 PUFA), there was no difference in any of the three p-wave parameters studied as a result of n-3 PUFA supplementation as shown in table 27. This is in keeping with the clinical outcome data in that n-3 PUFA did not reduce clinical AF.

Table-27: Effect of n-3 PUFA supplementation on P-wave parameters

Treatment group	**P-max (baseline)**	**P-max (at surgery)**	***p***
Placebo	107.4±10 (ms)	108.1±9.6	0.79
n-3 PUFA	106.7±10.3	106.4±10.4	0.92
	P-min (baseline)	**P-min (at surgery)**	
Placebo	80.9±6.6	78.8±7.9	0.48
n-3 PUFA	79.3±10	80.4±6.8	0.41
	PWD (baseline)	**PWD (at surgery)**	
Placebo	26.5±9.3	27.9±9.7	0.39
n-3 PUFA	28.8±11.3	26.0±9.7	0.46

3.1.9: HRV in the two groups (3rd post operative day)

Of the several heart rate variability parameters studied, the n-3 PUFA group showed a significantly higher high frequency (HFn) signals in the frequency domain analysis as detailed in table 28. This variable correlates with high parasympathetic tone. This difference was seen in the analysis of 1-hour recording of pacing and ectopic free traces of continuous ECG.

Table-28: Differences in HRV between control and n-3PUFA group

HRV parameter	Placebo	n-3PUFA	*p*
SDNNIDX	25.6±17.9	29.9±15.8	0.37
RMSSD	29.2±36.2	36.5±28.4	0.43
Triangle index	15.3±7.5	16.0±5.6	0.69
HFn	36.2±11.4	43.3±13.1	0.04
LFn	42.3±18.8	34.7±18.8	0.16
LF/HF	2.0±1.6	1.4±1.3	0.13

3.2: Q-PCR results

Of the 103 patients who were enrolled in the study, frozen right atrial appendage tissue from 61 patients (Placebo-31, n-3 PUFA 30) were used for extraction of c-DNA of which 52 (Placebo-26, n-3 PUFA-26) were used for Q-PCR analysis. An internal control sample (tissue obtained from a patient who was not enrolled into the study and who had not taken any n-3 PUFA in the 3 month period prior to surgery) was used in the Q-PCR reaction. The m-RNA values were expressed as relative expression of c-DNA compared to a house keeper gene (28s subunit protein) and an internal control sample (the delta-delta- Ct method). The mean values with error margins are shown in table-29 and the functional relevance of some the encoded proteins are shown in table-30.

The relative expression of c-DNA for individual group of ion channels, transporters and calcium handling proteins are depicted in figures 3.2 to 3.7. Among the calcium handling proteins, significant changes in expression levels were seen in total phospholamban with a lower expression in the n-3 PUFA group without a concomitant

change in SERCA expression. In addition, there was increased expression of ryanodine receptor protein in the n-3 PUFA group. It was noted that there was also a significantly higher expression of M2 receptor proteins in the n-3 PUFA group. The changes seen in other ion channels and transporters did not reach significance levels.

Table-29: Results of Q PCR (m-RNA levels) of various proteins in human RAA

Target protein tested	ΔΔCt values (mean ± SEM)		*P*
	Placebo (n=31)	n-3 PUFA (n=30)	
$Na_v1.5$	0.07± 0.01	0.04 ± 0.01	0.12
$Na_v\beta1$	0.16±0.03	0.23± 0.05	0.30
$Ca_v1.2$	0.08±0.01	0.13± 0.04	0.22
$Ca_v1.3$	0.11±0.03	0.13± 0.06	0.53
$K_v1.5$	0.23 ± 0.04	0.31 ± 0.06	0.29
$K_v4.3$	0.10±0.02	0.12±0.03	0.57
$K_{ir}2.1$	0.21±0.05	0.33±0.07	0.18
$K_{ir}3.1$	0.10±0.02	0.13±0.03	0.37
$K_{ir}3.4$	0.21±0.03	0.27±0.08	0.47
$K_{ir}6.2$	0.13±0.03	0.18±0.05	0.33
NCX1	0.17± 0.05	0.26±0.07	0.09
RYR2	0.11±0.02	0.27± 0.06	**0.04**
Phospholamban	0.20±0.04	0.10 ±0.02	**0.02**
SERCA2a	0.22 ±0.07	0.20±0.05	0.83
β1	0.17±0.04	0.13±0.02	0.60
β2	0.19±0.03	0.25±0.05	0.32
M2	0.09±0.02	0.19±0.04	**0.03**
Cx40	0.16± 0.04	0.21± 0.04	0.36
Cx43	0.25 ± 0.05	0.29 ±0.06	0.61

Table-30: Functional relevance of changes in m-RNA levels

Ionic current/ Function	**Ion channel**	**mRNA abundance ΔΔCt values (mean ± SEM) (Placebo)**	**mRNA abundance ΔΔCt values (mean ± SEM) (n-3 PUFA)**	***P (Unpaired t-test (or) Mann Whitney U-test)***
I_{Na}	$Na_v1.5$	0.07± 0.01	0.04 ± 0.01	0.12
I_{CaL}	$Ca_v1.2$	0.08±0.01	0.13± 0.04	0.22
I_{to}	$K_v4.3$	0.10±0.02	0.12±0.03	0.57
I_{Kur}	$K_v1.5$	0.23 ± 0.04	0.31 ± 0.06	0.29
I_{K1}	$K_{ir}2.1$	0.21±0.05	0.33±0.07	0.18
I_{KACH}	$K_{ir}3.1$	0.10±0.02	0.13±0.03	0.37
I_{KATP}	$K_{ir}6.2$	0.13±0.03	0.18±0.05	0.33
$I_{Na/Ca}$	NCX1	0.17± 0.05	0.26±0.07	0.30
SR calcium release	RyR2	0.11±0.02	0.26± 0.07	**0.04**
SR calcium uptake	SERCA2a	0.22 ±0.07	0.20±0.05	0.83
SERCA inhibition	PLB	0.20±0.04	0.10 ±0.02	**0.017**

Figure 3.2: Expression levels of m-RNA of calcium handling proteins

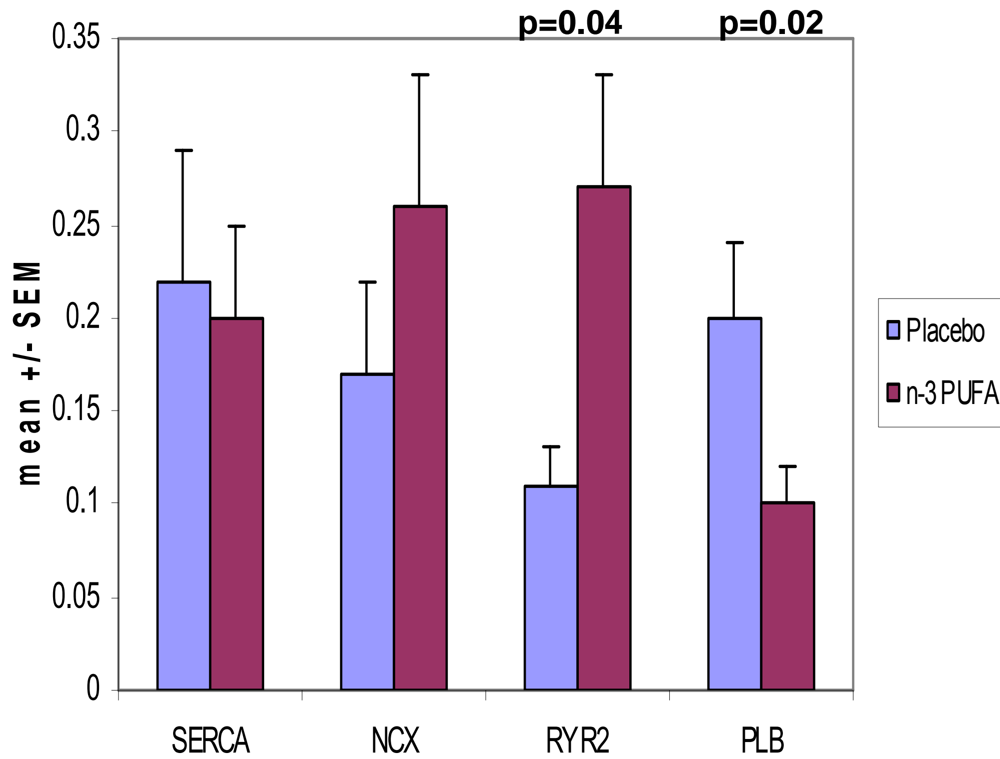


Figure 3.3: Expression levels of m-RNA of cardiac connexins

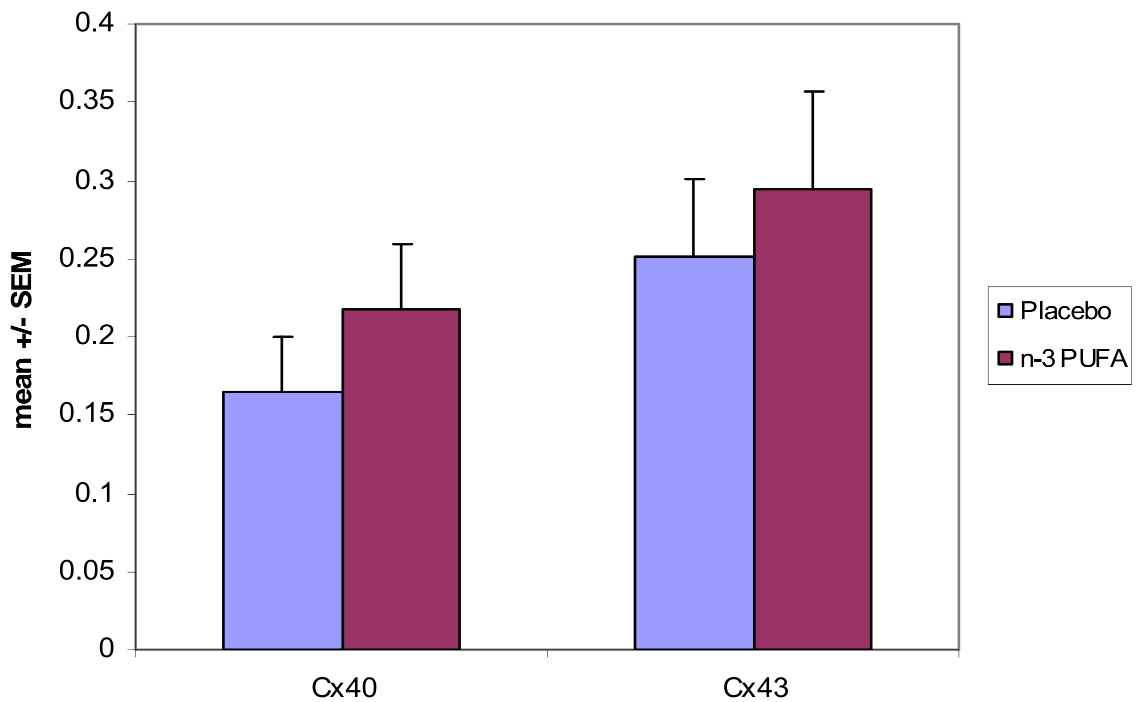


Figure 3.4: Expression level of m-RNA of cardiac autonomic receptors

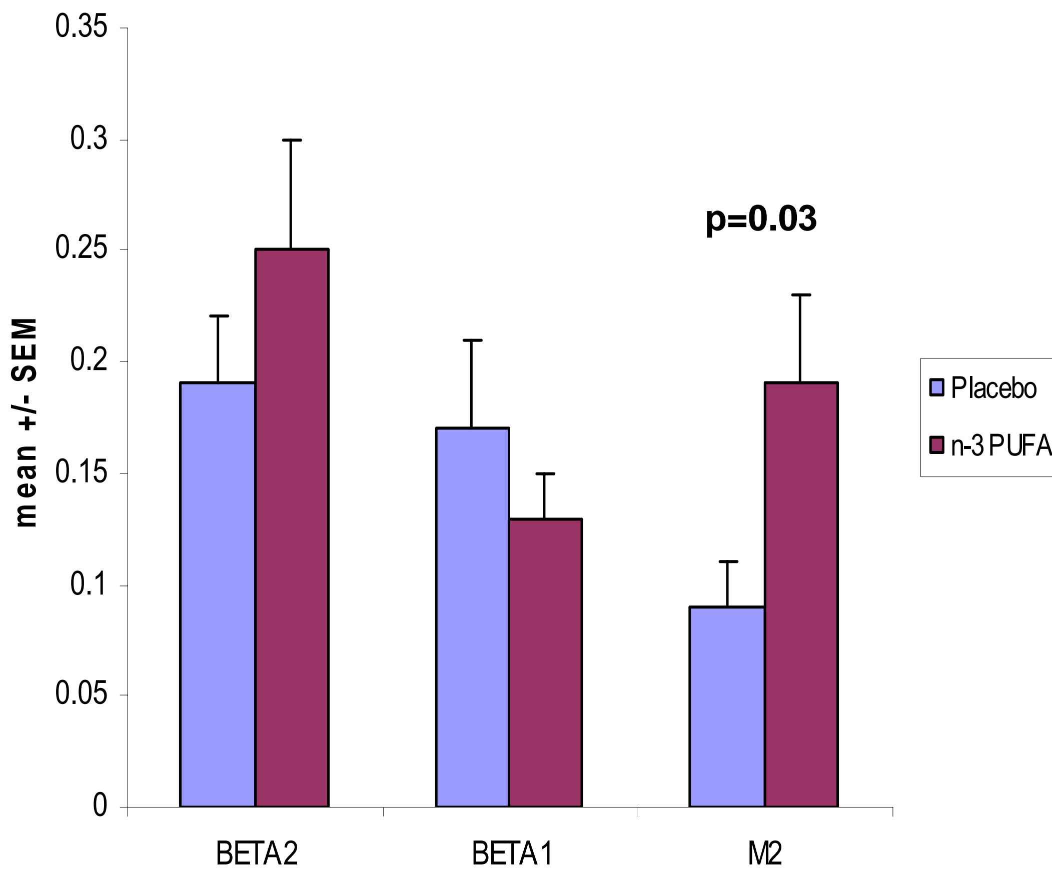


Figure 3.5: Expression level of m-RNA of cardiac sodium channels

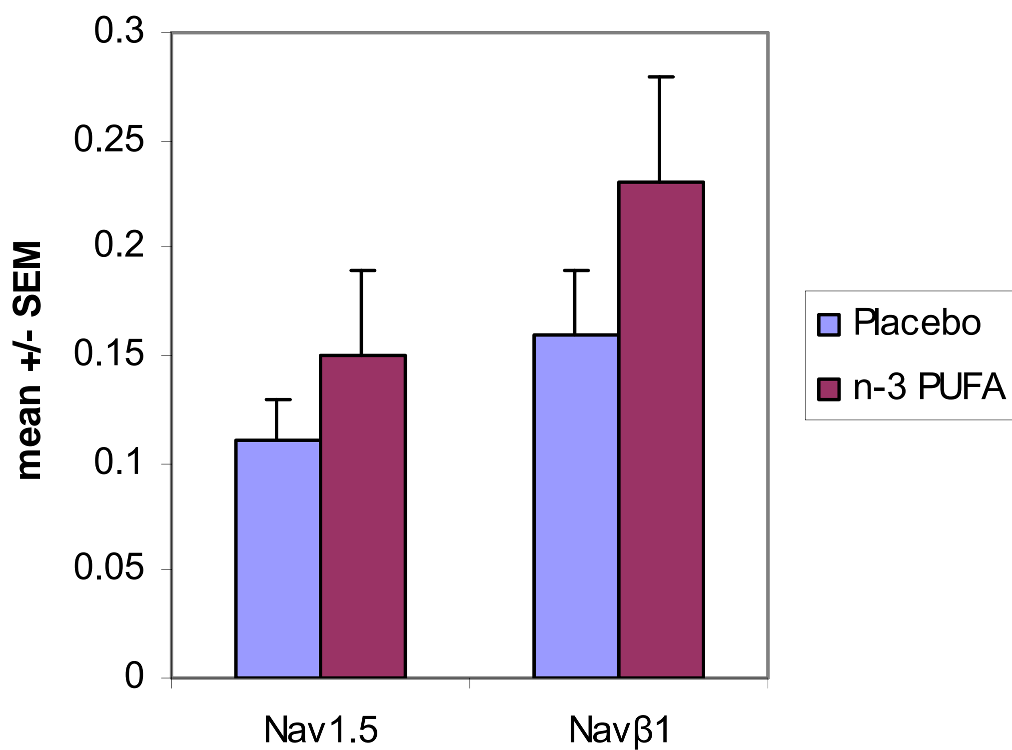


Figure 3.6 (A): Expression level of m-RNA of cardiac potassium channels

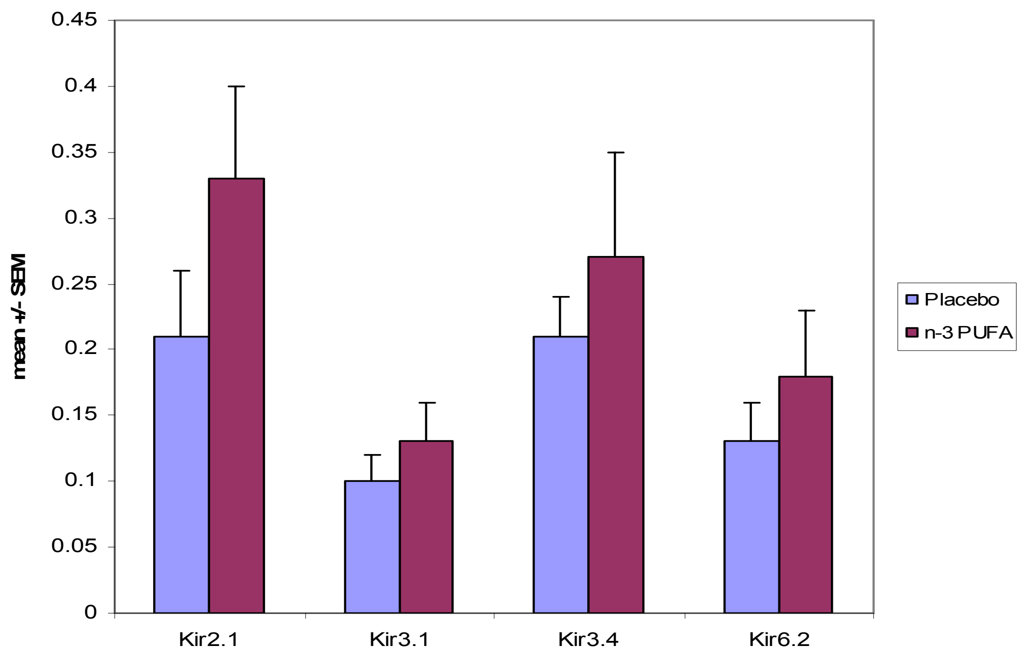


Figure 3.6 (B): Expression level of m-RNA of cardiac potassium channels (It_o and IK_{ur})

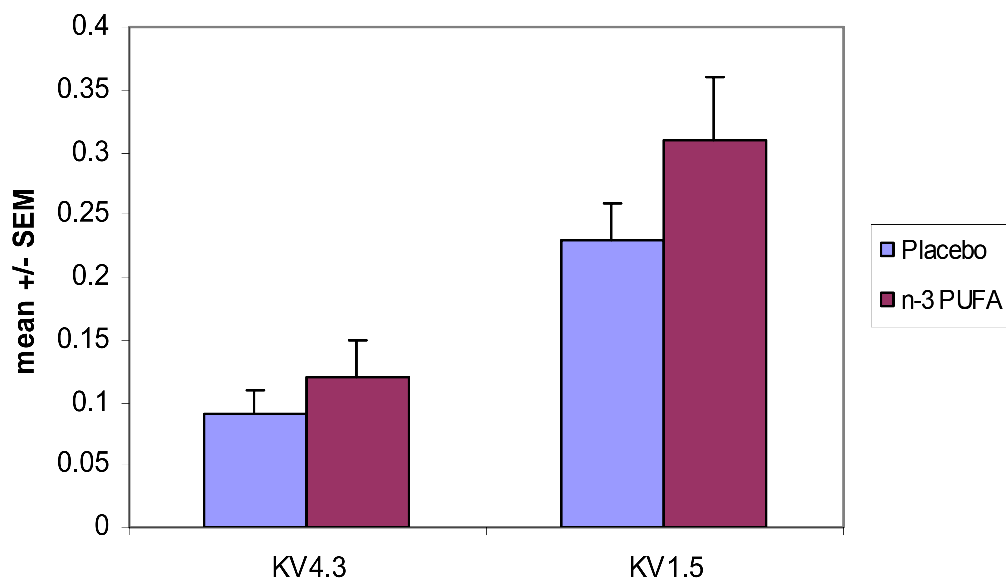
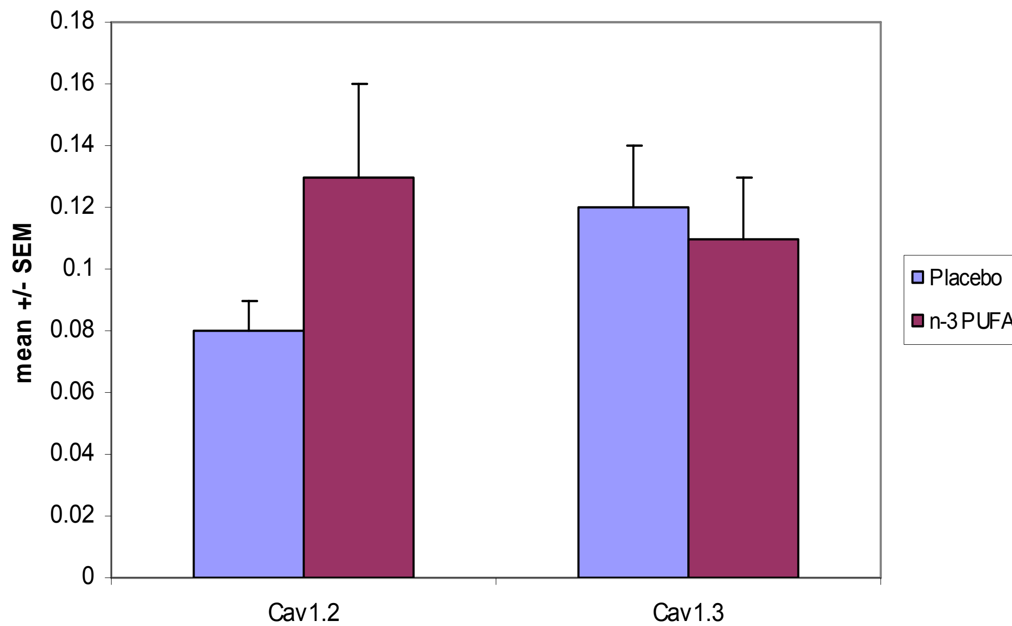


Figure 3.7: Expression level of cardiac calcium channels



3.3: Immunohistochemistry-Results

The role of immunohistochemistry was mainly to evaluate the distribution pattern of a target protein, however, semi-quantitative estimates of the fluorescence emitted (as measured by a specifically designed velocity soft ware package) could be used to evaluate the expression level of a given target protein. This method has been reliably used in several studies within our group and other workers in this field. The mean fluorescence was measured for calcium handling proteins and is shown in table-31 and figure-3.8.

Table-31: Semi-quantitative assessment of expression of calcium handling proteins in Immunohistochemistry.

Target protein	Mean Fluorescence- (mean ± SEM)		*p*
	Placebo (n=8)	n-3 PUFA (n=8)	
Caveolin	42.9±1.1	36.5±3.8	0.25
NCX	55.9±4.17	68.2±4.4	0.07
SERCA	77.5±3.9	81.3±3.6	0.50
Phospholamban	80.6±4.5	59.5±2.6	0.002
RYR-2	70.7±3.1	85.9±3.3	0.01

Figure 3.8: Immunofluorescence estimates of calcium handling proteins

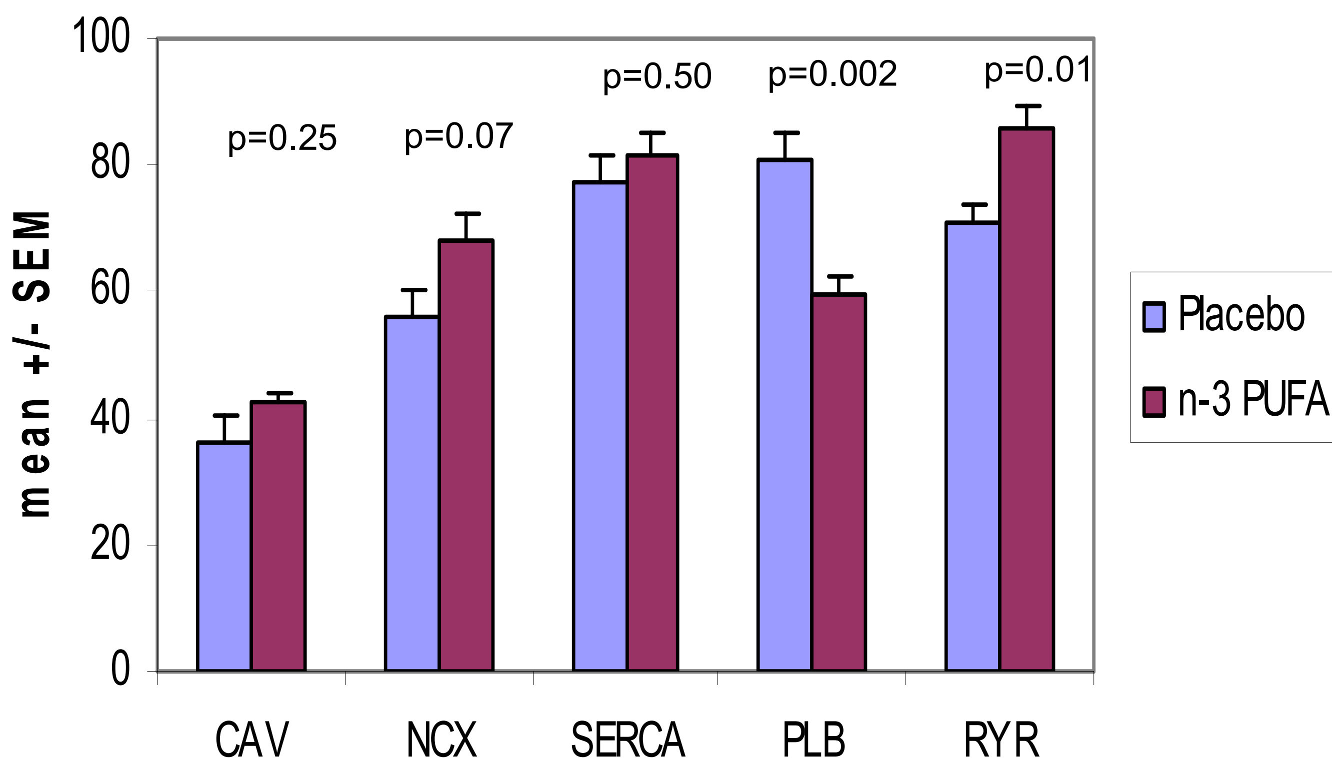


Figure 3.9: Immunohistochemistry- representative images of caveolin expression.

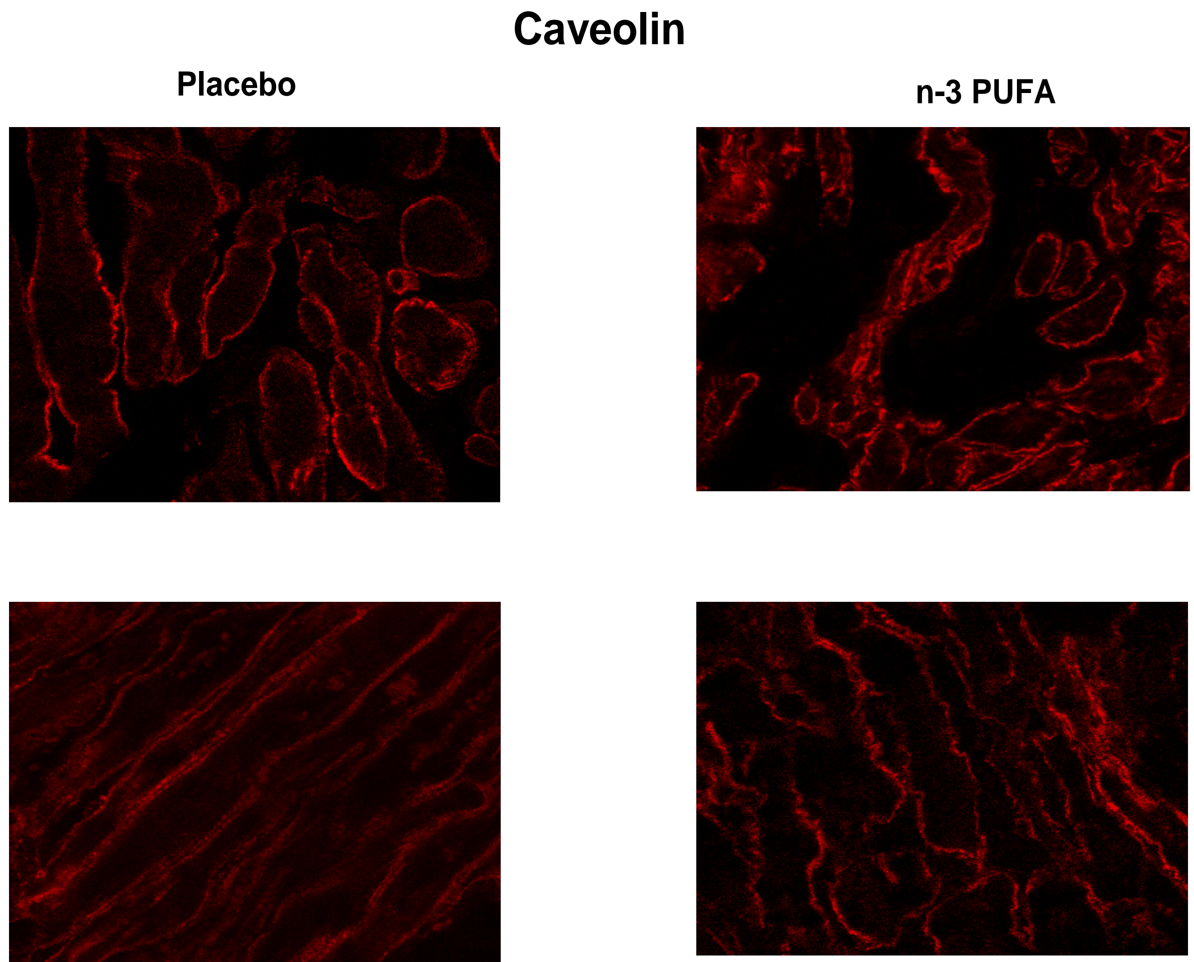


Figure 3.10: Immunohistochemistry- representative images of NCX expression

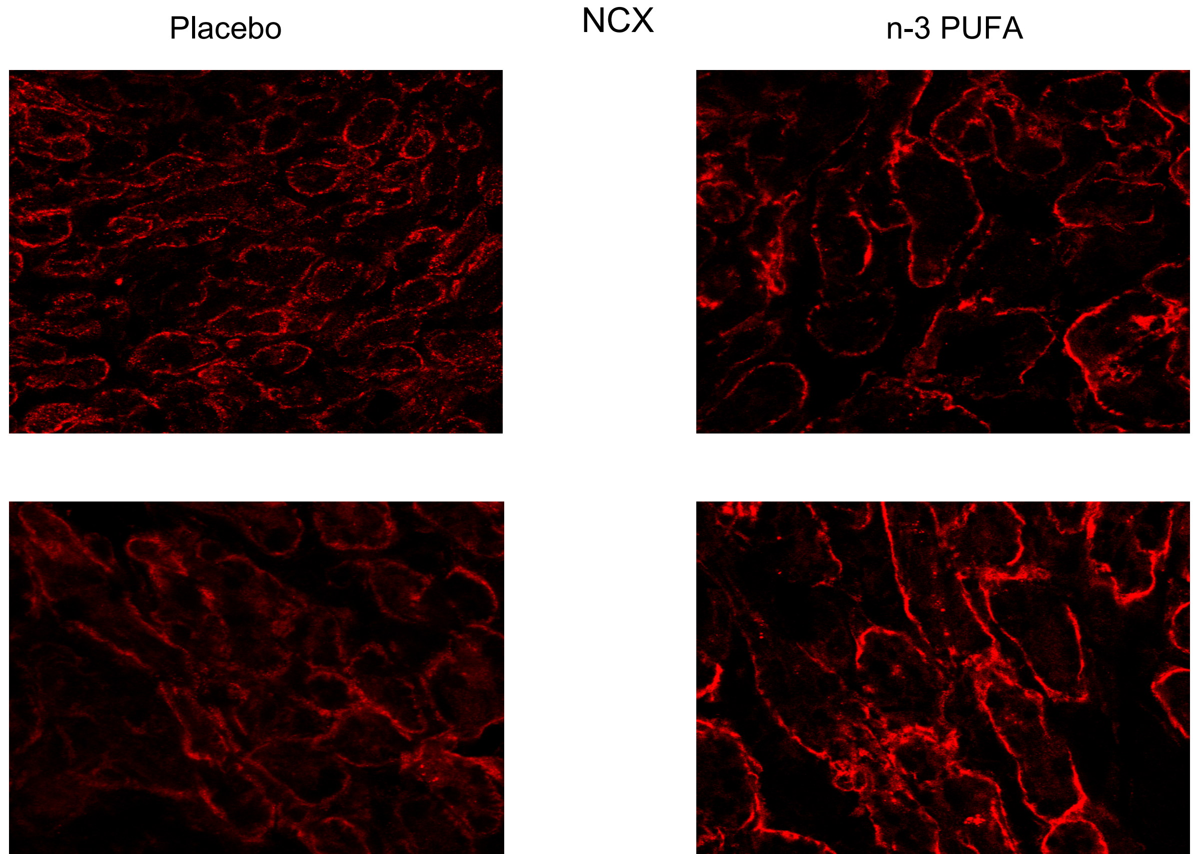


Figure 3.11: Immunohistochemistry- representative images of SERCA expression

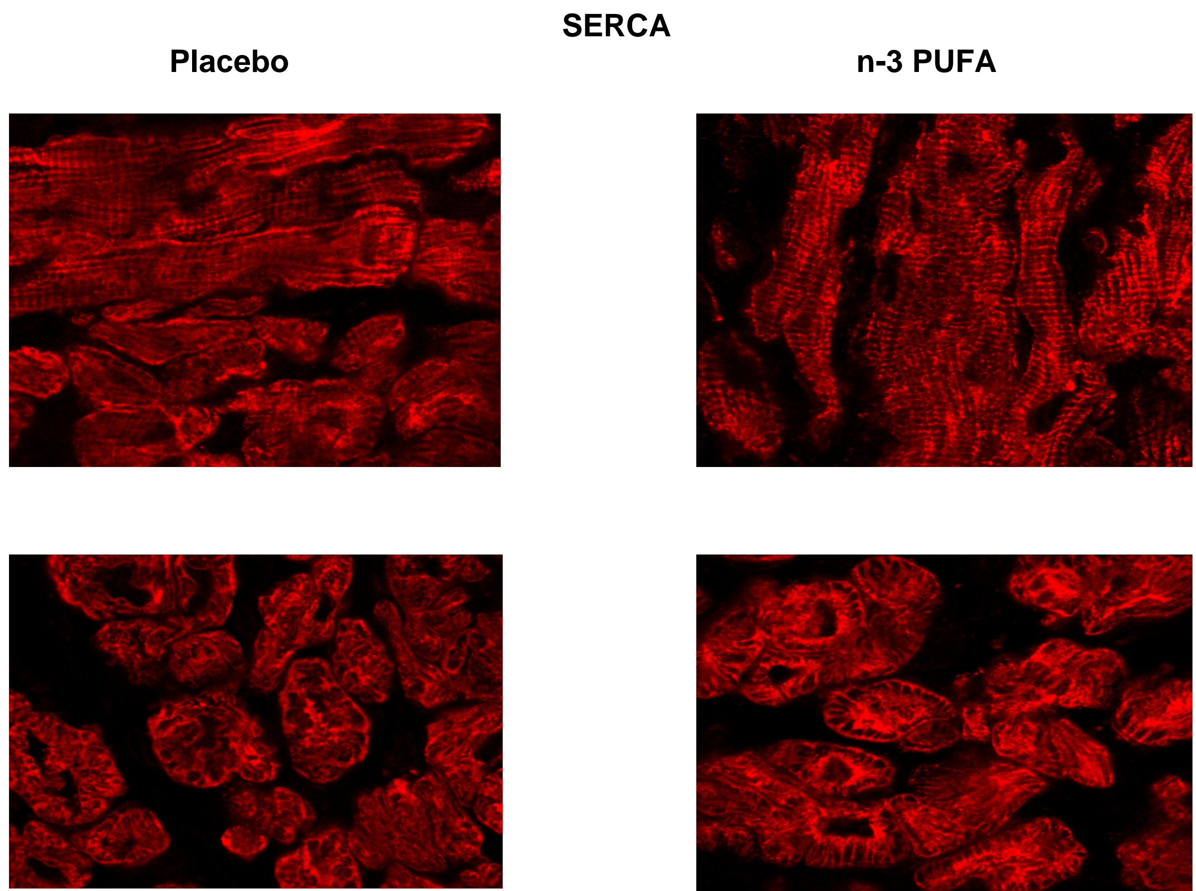


Figure 3.12: Immunohistochemistry- representative images of phospholamban expression

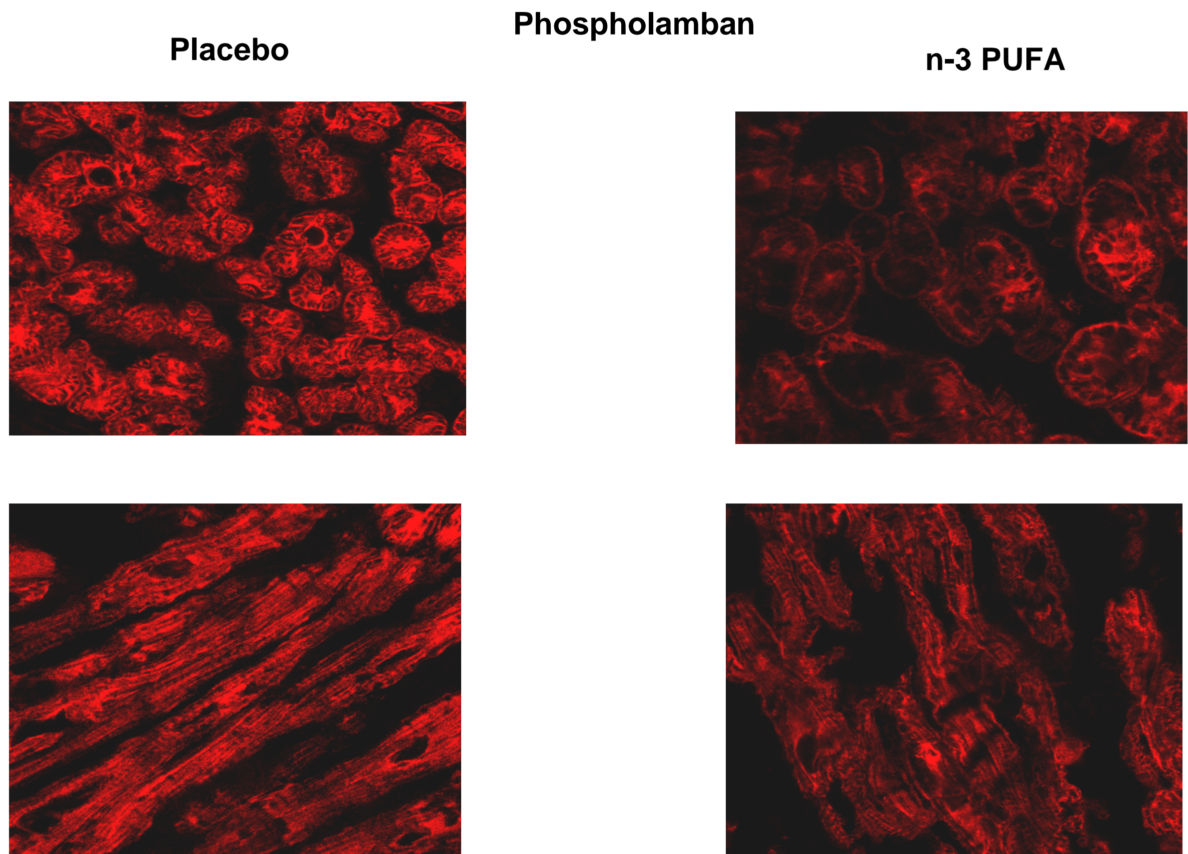
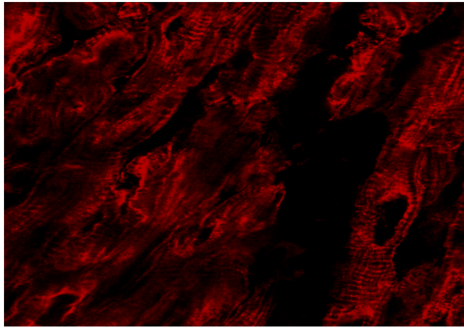
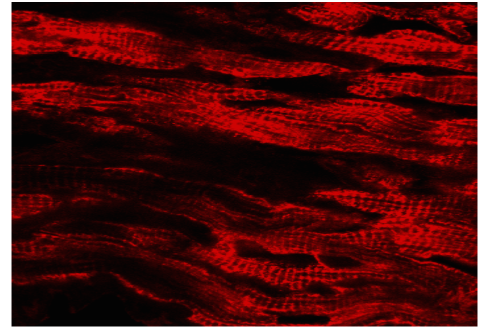
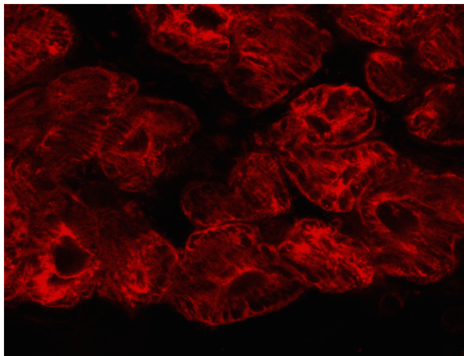
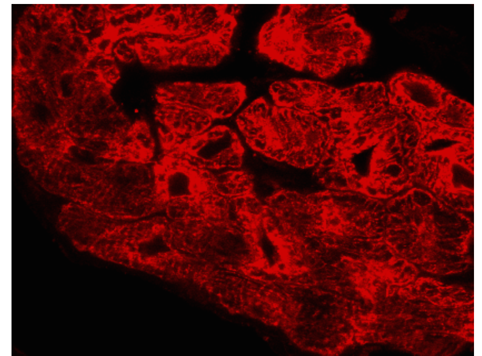


Figure 3.13: Immunohistochemistry- representative images of RYR2 expression

RYANODINE RECEPTOR

Placebo	n-3 PUFA
	
	

3.4: Western blot- Results

In addition to qPCR and immuno-histochemistry, accurate quantification of expression of calcium handling proteins was attempted using Western blot technique. The resultant chemi-luminescence (a measure of the quantity of the target protein found in a given quantity of protein concentrate) was normalised for a loading control to correct for pipetting error and was again normalised for an internal control sample which was used in all the reactions so that uniformity of measurement between reactions could be ensured. The resultant mean values are given in table 32 and figure 3.14.

Table 32: Western blot- Expression of calcium handling proteins

	Placebo (n=8)	**n-3 PUFA (n=8)**	***p***
NCX	1.01±0.10	1.05±0.14	0.85
SERCA	1.20±0.09	1.13±0.11	0.60
PLB	0.91±0.07	0.70±0.06	**0.04**
RYR2	0.87±0.12	0.93±0.24	0.83

Figure 3.14: Western blot- relative abundance of calcium handling proteins

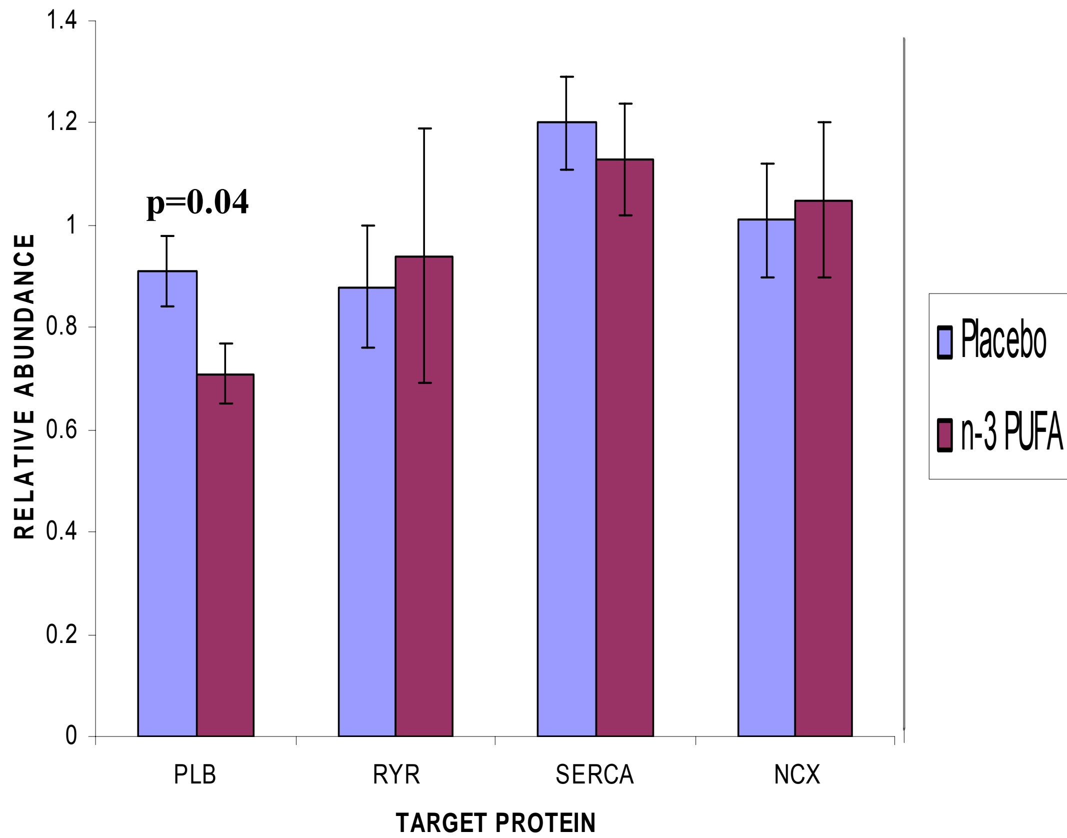
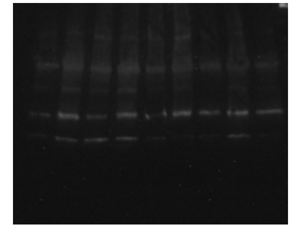
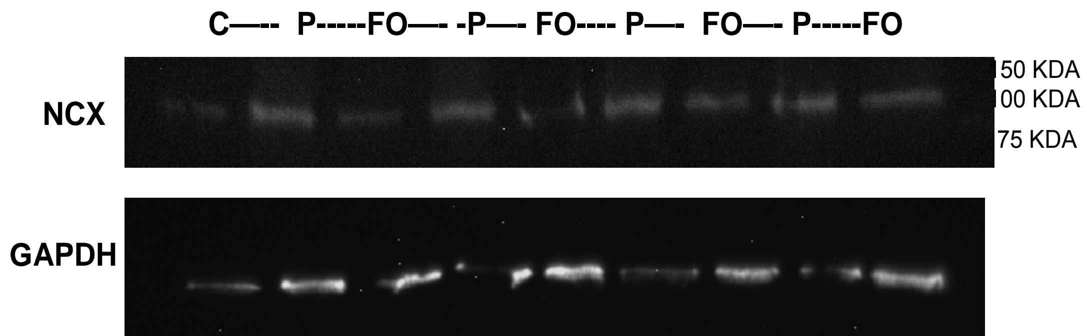


Figure 3.15: Western blot- expression of NCX- representative image

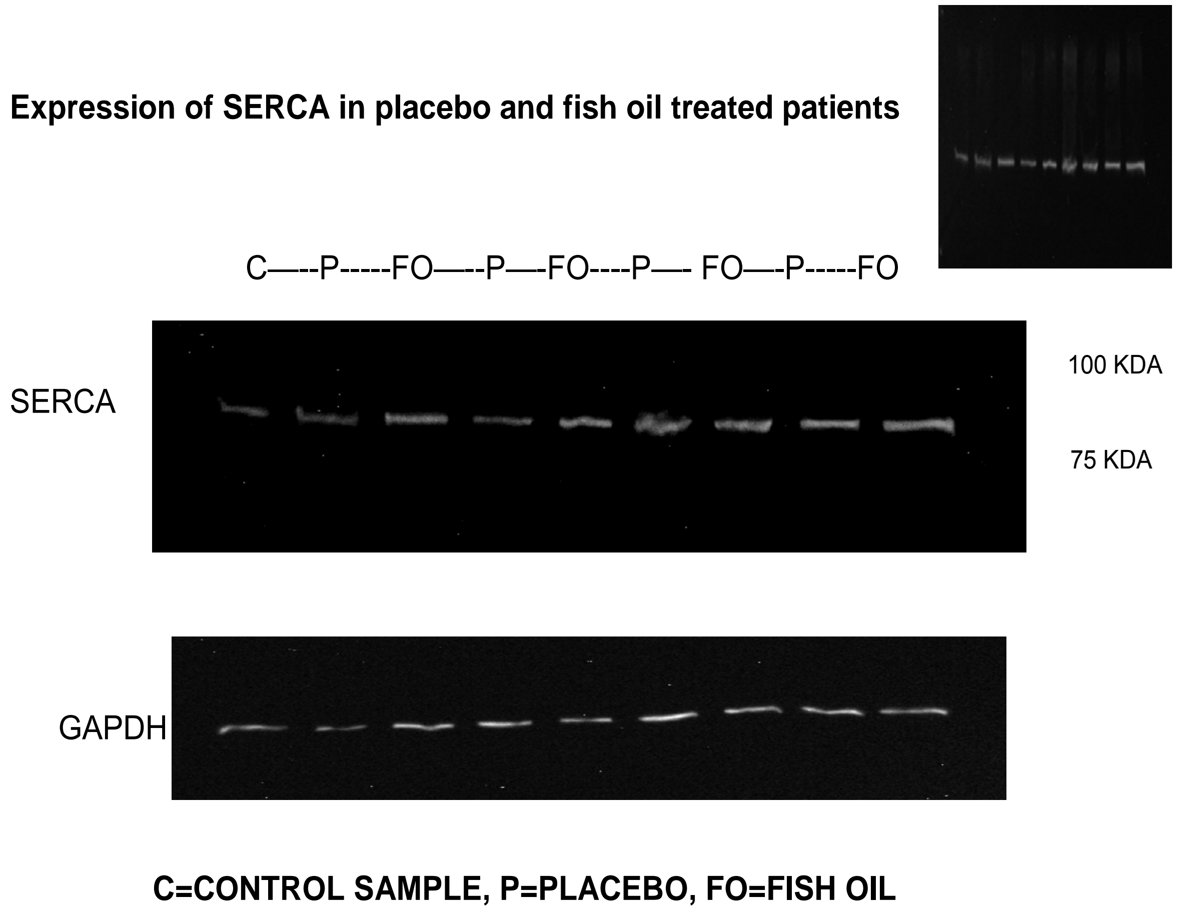
Expression of NCX in placebo and fish oil treated patients





C=CONTROL SAMPLE, P=PLACEBO, FO=FISH OIL

Figure 3.16: Western blot- expression of SERCA- representative image



C=CONTROL SAMPLE, P=PLACEBO, FO=FISH OIL

Figure 3.17: Western blot- expression of PLBA2- representative image

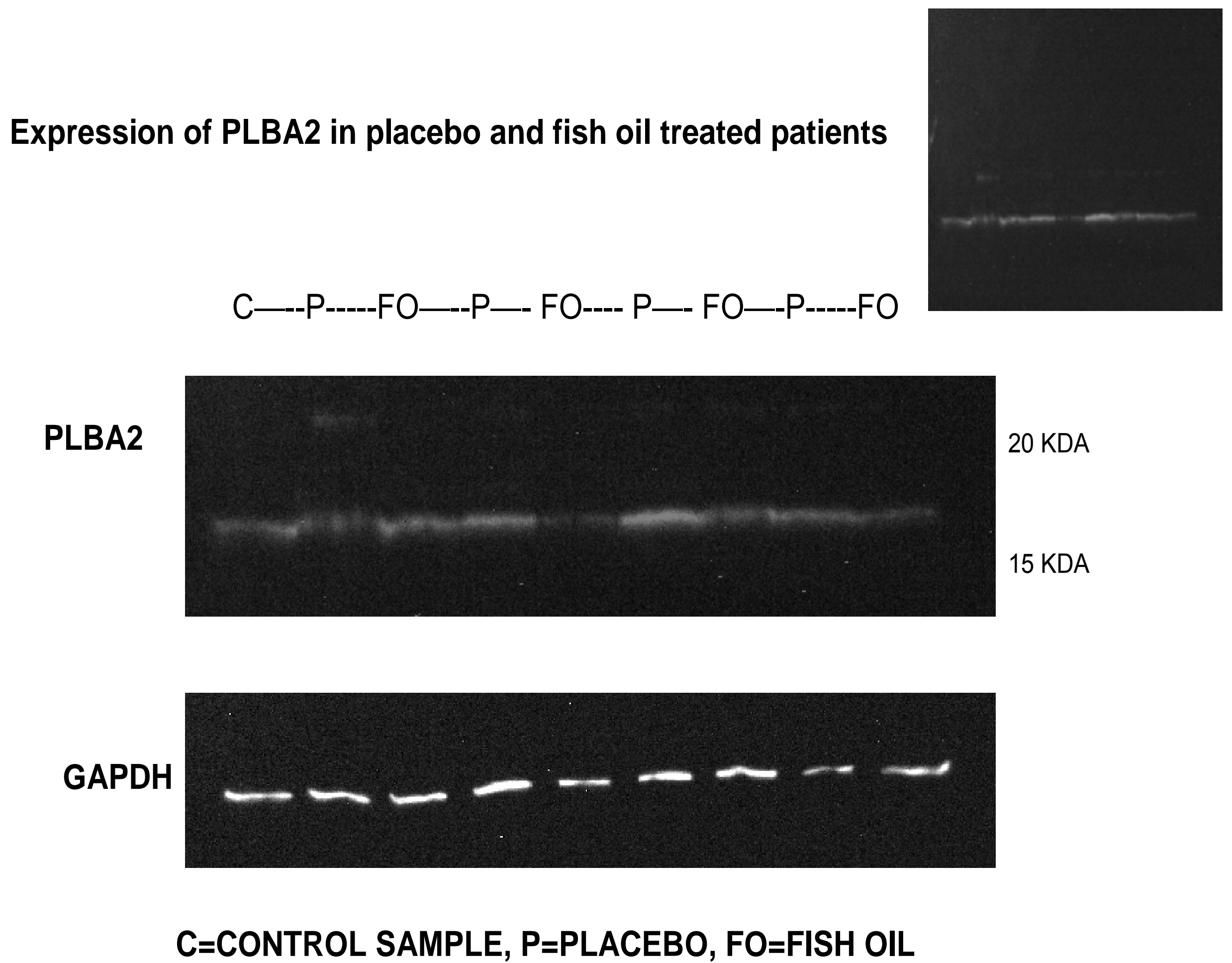
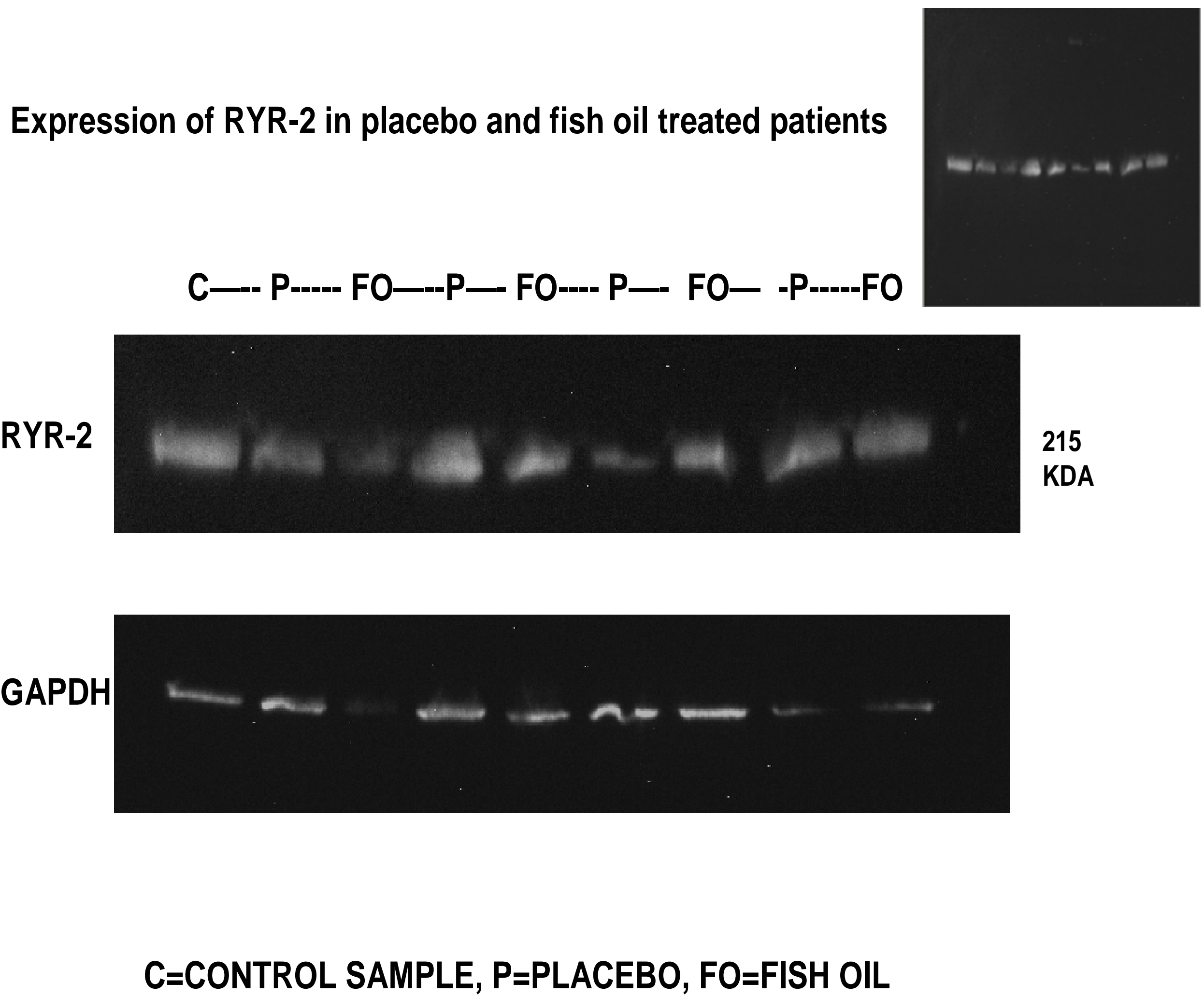


Figure 3.18: Western blot- expression of RYR2- representative image



3.5: Animal Experiments (isolated rat ventricular myocytes)-Results

The effect of n-3 PUFA incorporation in the membrane phospholipids on the calcium handling properties of these cells were evaluated by performing field stimulated experiments on isolated rat ventricular myocytes loaded with fluo-3M (a calcium sensitive dye) and compared with similar cells from animals fed a standard chow. The frequency of stimulation was 0.5Hz throughout the experiments.

3.5.1: Calcium transient amplitude

Having shown differences in the expression level of calcium handling proteins, we proceeded to evaluate the functional relevance of such a change. Unfortunately, we could not consistently obtain good quality myocytes from human atrial appendage tissue as described in chapter-2. Therefore we performed these experiments on isolated ventricular myocytes from rats fed n-3 PUFA supplemented diet and compared it with those fed standard chow. Field stimulation experiments were carried out as described before to obtain Ca^{+} transient amplitude of the rat ventricular myocytes. In some cases the cells were not accurately calibrated to calcium due to a failure to obtain a maximal fluorescence for fluo-3F (Fmax) measurement at the end of the experiment. Therefore, intracellular calcium is expressed as F/F_0, where F is the fluorescence measurement and F_0 is fluorescence at diastolic levels, after subtracting background fluorescence. Background fluorescence is determined by the measurement of fluorescence in the absence of a cell. However, in all the cells where Fmax was available, both actual calibration of calcium and F/F_0 was estimated. The mean data of the F/F_0 estimation of the calcium transient amplitude shows that the calcium transient amplitude of the ventricular myocytes is not significantly different between the two groups as shown in table-34. (2.44 ± 0.11 in control (n=17) Vs 2.51 ± 0.09 in n-3 PUFA (n=16) (F/F_0), P=0.71).

Table 33: Amplitude of systolic calcium transient (F/F_0)

	Control	**n-3 PUFA**	***p***
Ca^+ transient Amplitude (F/F_0)	2.44 ± 0.11	2.51 ± 0.09	0.71

3.5.2: Rate of decay of systolic calcium transient

The rate of decay of the systolic calcium transient was measured as the time taken to decay from 90% of the transient amplitude to 50% of the amplitude. The decay was significantly faster in the n-3 PUFA treated ventricular myocytes as shown in table-35 and figure 3.19. Rates of decay of the calcium transients were plotted using an exponential curve fit with the use of purpose built software (in-house) modified from Microsoft-Excel- Visual Basic software package. A representative curve fit demonstrating a faster rate of decay in a n-3 PUFA treated ventricular myocyte compared to a control myocyte is shown in figure-3.20.

Table-34: Time taken for the decay of systolic calcium transient from 90% to 50% amplitude

	Control	**n-3 PUFA**	***p***
Time to decay from 90% to 50% transient amplitude (mean ± SEM)	170.7±47.7	128.7±69.2	0.02

Figure 3.19: Rate of decay of systolic calcium transient in n-3 PUFA treated myocytes compared to control myocytes

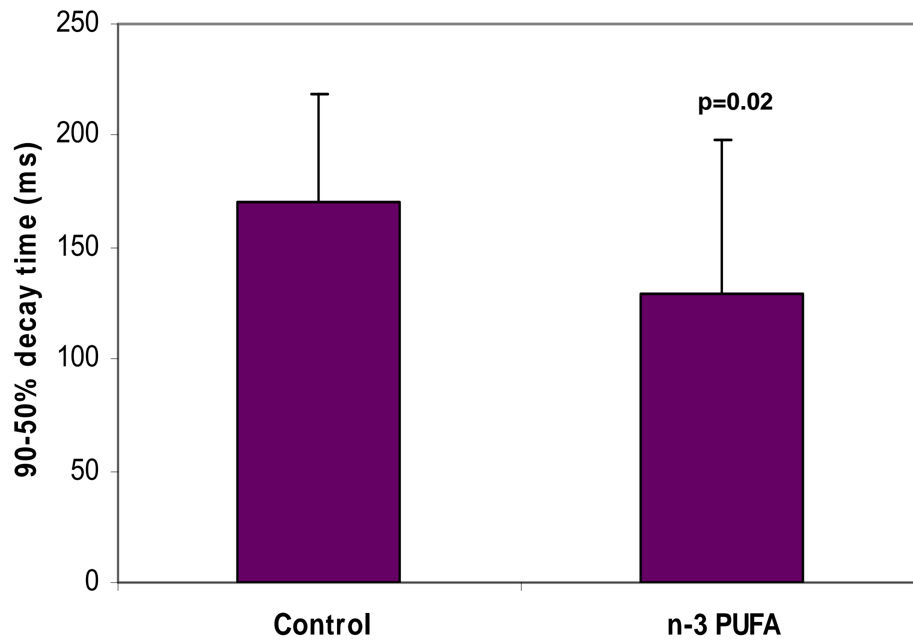
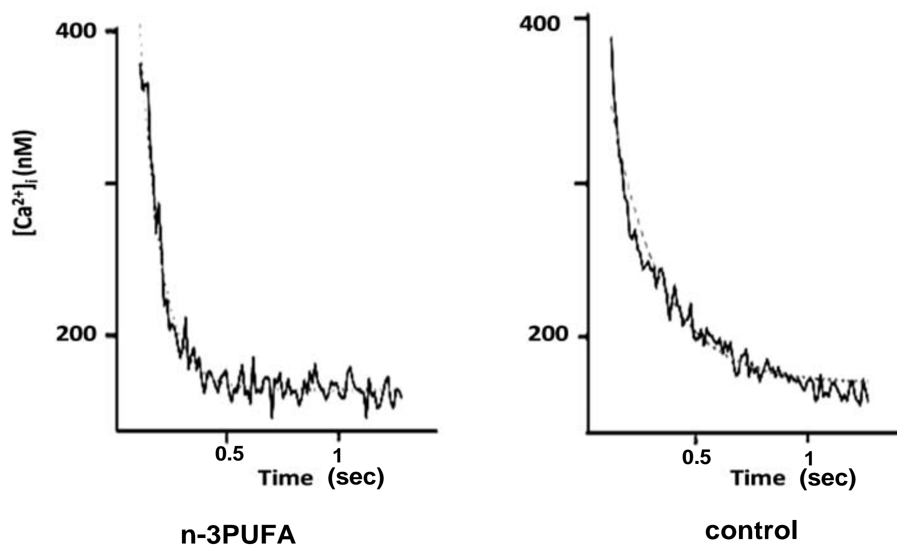


Figure 3.20: Representative image showing a faster rate of decay of systolic calcium transient in n-3 PUFA treated myocytes.



3.5.3: SR calcium content

Application of 10mM caffeine discharges the SR calcium store and a measure of the calcium transient thus obtained is an estimate of the SR calcium content. In the same field stimulation experiment, following a series of stimulus to obtain a steady systolic transient, stimulation was stopped and 10mM caffeine applied as shown in a representative image depicted in figure 3.21. The resultant transient was recorded in both groups to assess the effect of n-3 PUFA supplementation on the calcium content of the cell as SR is the main storage site for intra-cellular calcium. Again, as we did not have Fmax values in some experiments, to maintain uniformity of data, F/F_0 was used to estimate the amplitude of the caffeine transient. This showed that the calcium transient obtained after caffeine application was significantly higher in the n-3 PUFA treated cardiomyocytes as shown in figure 3.22. The mean values of F/F_0 for the caffeine transients were 4.22±0.86 for the control cells Vs 5.61±0.43 for n-3 PUFA treated cells (p=0.03, Student's t-test)

Figure 3.21: A typical experimental trace (control cell) illustrating systolic calcium transients followed by application of 10mM caffeine to release total SR calcium and a gradual return to steady state

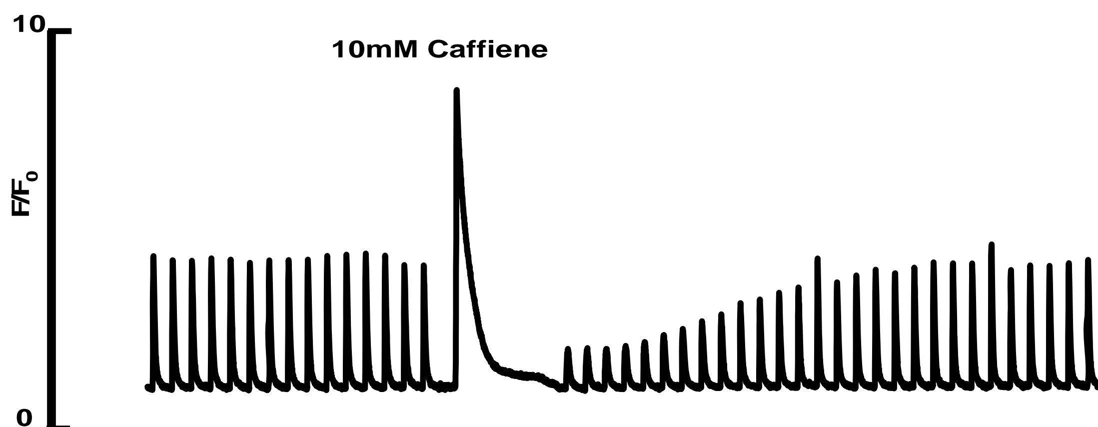
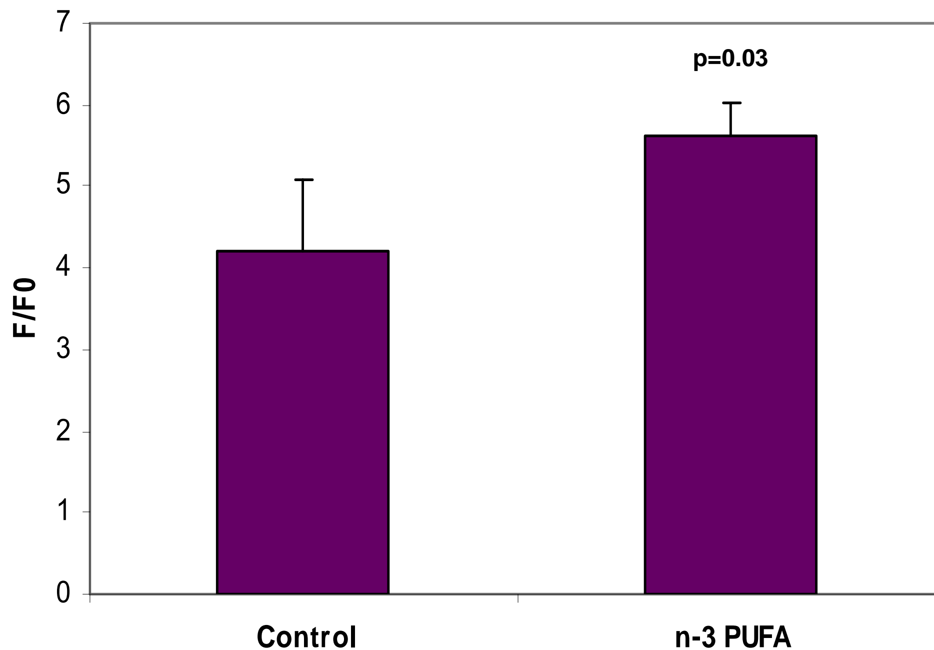


Figure 3.22: Mean amplitude of caffeine transient (F/F_0) in n-3 PUFA Vs control cells



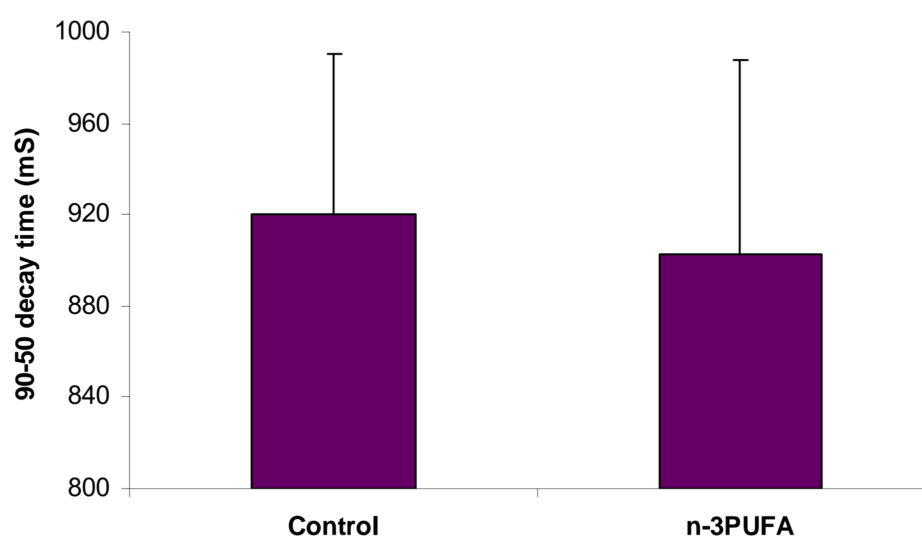
3.5.4: Intracellular and sarcolemmal calcium removal mechanisms.

Calcium removal occurs via the intracellular calcium pump, SERCA, and through sarcolemmal (SL) calcium transporters, NCX and PMCA. Application of caffeine completely inhibits SERCA activity and therefore the rate of decay of the calcium transient obtained after application of caffeine is a sum of the measure of activity of the other 2 transporters- NCX and PMCA. As shown above, the rate of decay (as measured by the time taken to decay from 90% to 50% fluorescence) during the normal systolic calcium transient is significantly faster in the n-3 PUFA treated cardiomyocytes compared with the control myocytes. However the rate of decay of caffeine evoked calcium transient (again as measured by the time taken to decay from 90% to 50%) was not altered by n-3 PUFA therapy as shown in table-35 and figure 3.23.

Table 35: Rate of decay of caffeine transient

	Control	n-3 PUFA	p
Time to decay from 90% to 50% amplitude (mean ± SEM)	919.7±70.4	902.7±84.9	0.66

Figure 3.23: Rate of decay of caffeine evoked calcium transient in n-3 PUFA treated cells compared to control myocytes.



Chapter 4

Chapter-4: Discussion

4.1: Clinical Outcomes

4.1.1 n-3 PUFA and AF following CABG

The main finding of this study is that short term therapy with n-3 PUFA raises levels of EPA and DHA in serum and phospholipids of the cell membranes of circulating PBMC and atrial tissue, but does not reduce the incidence of AF following CABG. It was also found that by continuous monitoring of ECG, the overall incidence of significant AF episodes (defined as any AF ≥ 30 sec) was higher than most previously published reports [21,22]. It could be argued that the criteria for diagnosing AF in our study was strict and the monitoring robust such that some patients would have had very brief AF episodes lasting for a short duration which would otherwise not have received any attention. If this was true, the clinical relevance of such episodes may also be questioned. In order to address these issues, we had prospectively planned to look at 2 variables as secondary end points, both of which would clearly focus on this question and aim to give an unambiguous answer.

1) AF burden- Defined as the period of time a given patient was in AF in a 24 hour period, expressed as a percentage. By this method we were able to identify the total duration of AF/day/patient and the shortest of this was 0.7hrs (42 minutes), which would clearly have clinical relevance in terms of symptoms and/ or risk of thrombo-embolic episodes. In this respect, a recent study evaluating the risk of thrombo-embolism in patients with atrial arrhythmias and an implanted device [193] (pacemaker or defibrillator) concluded that an AF burden of ≥5.5hrs over a 30 day period of monitoring indicated high risk of thrombo-embolic episodes. If we extrapolate this to a short duration of monitoring in our patients (5 days) then it

would not be unreasonable to suggest that the AF burden seen in the cohort of patients who experienced post operative AF could have pre-disposed these patients to a high risk of thrombo-embolic strokes even though, fortunately, there were no thrombo-embolic events in the entire study group.

2) The other secondary outcome measure that addressed this issue is that of clinically identified (and on most occasions required specific therapy) AF which was identified and documented by the treating physician/ surgeon. Whilst the incidence of clinically identified and treated AF episodes were fewer than that identified by the continuous monitoring, the incidence of such arrhythmic episodes were not different between the two groups, further confirming that n-3 PUFA did not exert a beneficial effect on the incidence of AF following CABG.

These findings contrast with those of Calo et al.(9), which was the first ever randomized clinical trial to evaluate the effect of n-3 PUFA on atrial fibrillation. This study reported a significant reduction in AF following CABG. At the inception of our study (2006) the study by Calo et al was the only clinical trial that had been published and we powered our study to show similar reduction in AF incidence. There were 2 major issues with the design of the study by Calo et al 1) It was an open label study with a potential for bias and 2) The method of arrhythmia monitoring was not robust as they relied on the daily review of a 3 lead ECG stored in the bed side monitor, which has a potential to miss short spells of arrhythmias along with a confounding influence of artefacts. Our aim, when we designed the study was to overcome these 2 potential limitations by a study that was randomised and double blinded along with a robust method for arrhythmia monitoring. In addition, we designed the study in such a way that the final outcome of AF will be adjudicated by a data monitoring committee who will not be a part of the research team

and this committee will review the data prior to recommending that the study should be concluded while the researchers were still blinded to the outcome data. At inception of our clinical study, we sought to confirm (or refute) the findings of the study by Calo et al and to perform cellular studies to evaluate changes at the cellular level that would explain such a beneficial/ harmful effect. Through the course of our study, we were aware of another study by Heidt et al [137] that was underway addressing the same question in a slightly different format. In their study, Heidt et al were administering intra-venous n-3PUFA suspension following CABG surgery in an open label fashion. In 2009, Heidt et al also reported a beneficial effect of n-3 PUFA administration in the immediate post-operative period however the methodological limitations of the study by Calo et al applied to that by Heidt et al in that both studies were open label studies and relied on less robust methods of arrhythmia monitoring.

Our findings were in clear contrast with the study by Calo et al and that by Heidt et al in that there was no evidence of any benefit with n-3 PUFA therapy and there could have been a potential harm but the study sample size was not adequate to conclusively address this issue as we had powered the study to show (or not) a benefit of the magnitude seen in the study by Calo et al. As our study design was more robust and we had very reliable data on the serum and membrane phospholipids levels of n-3 PUFA incorporation, we argued that our findings are more reliable. The other issues that could have influenced the overall outcome could be patient characteristics and concomitant drug therapy which even though was not different between the groups was significantly different from the study population in the study by Calo et and Heidt et al. Hence we performed a multiple logistic regression analysis to exclude the confounding influence of any of these factors and after

such analysis, we could confidently conclude that n-3 PUFA supplementation did not reduce the incidence of AF following CABG surgery in our study population.

Another important caveat is the dietary intake of n-3 PUFA as there is some evidence to suggest that over all higher dietary intake in some communities could reduce the magnitude of the influence of supplements. However, our dietary questionnaire clearly demonstrates that our cohort actually had a much lower dietary intake than that reported as baseline mean dietary intake in many observational and epidemiological studies [(64,76)]. Hence the dietary intake of n-3 PUFA in the overall study population could not have reduced the magnitude of beneficial effect in our study population.

4.1.2: Summary of clinical studies on the role of n-3 PUFA in AF

Since the publication of our study, 2 other studies have addressed the same question in a similar study design and have come to the same conclusion in that oral n-3 PUFA supplementation did not reduce the incidence of AF following CABG [(138,139)]. These studies lend credence to our findings.

A recent meta-analysis by Liu T et al [(140)] addressed the question of the effect of n-3 PUFA supplementation on the incidence of AF in 2 different clinical settings

1) AF following cardiac surgery

2) Recurrence of AF following cardioversion in patients with clinical persistent AF.

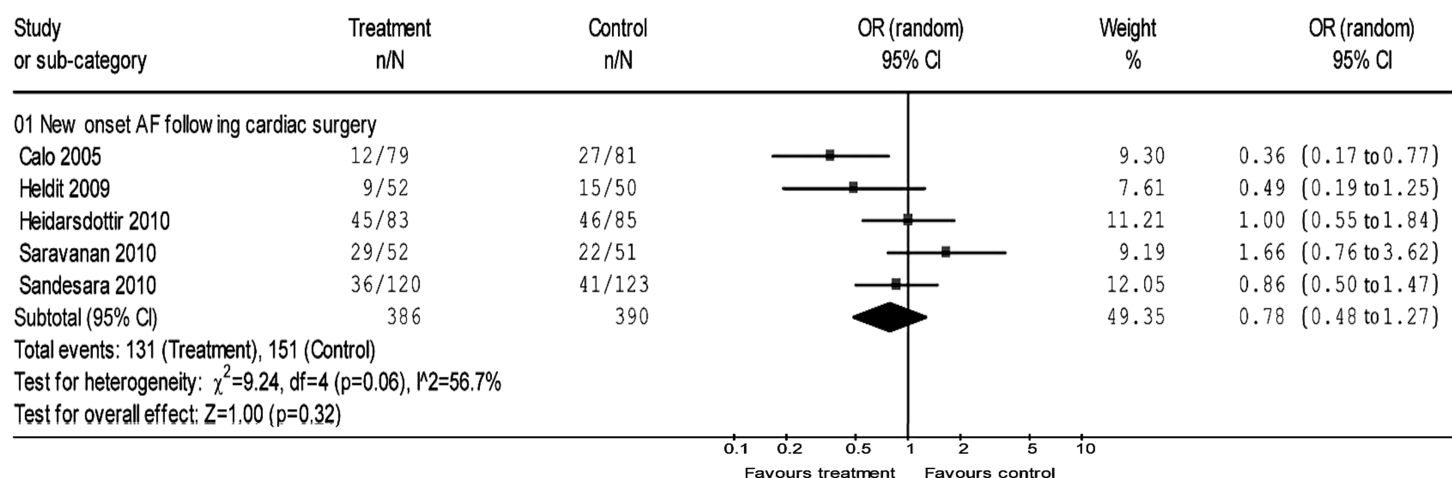
Data from 10 previously published studies, as shown in table-36, which evaluated the effect of n-3 PUFA on either of these two clinical settings was used in this analysis. The over all effect of n-3 PUFA supplementation on the incidence of AF of any form was not in favour of a beneficial effect.

This analysis used 5 clinical trials (of which our study is one) [9,137,138,139 &141] evaluating the risk of AF following CABG and convincingly showed that n-3 PUFA therapy did not reduce the risk of AF following CABG as shown in Figure 4.1. Interestingly, 2 [9,137] of the 5 studies were open label studies and both of them showed a significant reduction in the incidence of AF while the other three studies (including our study) were blinded studies and clearly demonstrated a lack of benefit. This suggests that observer bias could have been an important factor in influencing the outcome of studies that reported a beneficial effect.

Table 36: Summary of studies on the effect of n-3 PUFA on AF (modified from Liu et al).

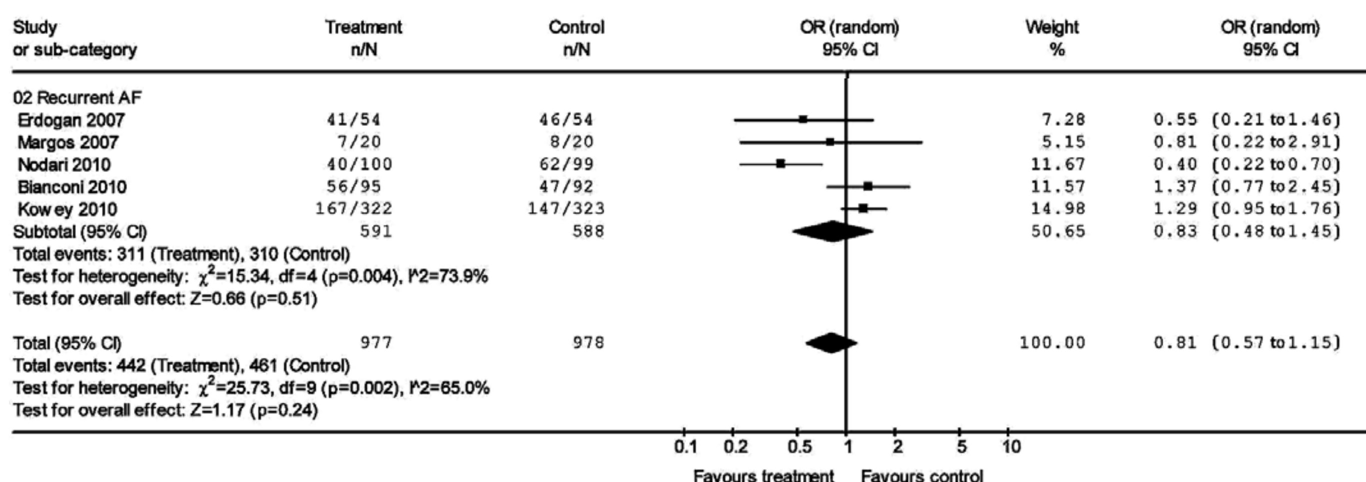
Author (study) year (ref no)	Country	Design	Study population	Regimen	No of patients	Dosage	Duration	Age (years)	Men (%
Calo *et al* 2005	Italy	R, OL	Post-CABG, SR	Intervention	79	0.85–0.88 g/day EPA; 1.7–1.76 g/day DHA	Immediate postoperative period until discharge	66.2	86
				Control	81	Usual care		64.9	84
Erdogan *et al* 2007	Germany	R, DB, PC	Persistent AF >48 h Post-cardioversion	Intervention	54	301.5 mg α-linolenacid	4 Weeks before and 1 year after EC	66.5	70
				Placebo	54	Placebo		63.5	74
Margos *et al* 2007	Greece	R, OL	Persistent AF Post-cardioversion	Intervention	20	PUFA	NA	54	85
				Control	20	Control		57	55
Heidt *et al* 2009	Germany	R, DB	Post-CABG, SR	Intervention	52	100 mg/kg per day PUFA infused by perfusion pump (EPA:DHA 0.9:1)	12 h before CABG until transfer from the ICU to a normal ward	65	73
				Control	50	100 mg/kg per day soya oil infused by perfusion pump		67	64
Heidarsdottir *et al* 2010	Iceland	R, DB, PC	Post-open heart surgery, SR	Intervention	83	1.24 g/day EPA; 1 g/day DHA	5–7 Days before surgery until discharge	67	81.9
				Placebo	85	2 g/day olive oil		67	76.9
Saravanan *et al* 2010	UK	R, DB, PC	Post-CABG, SR	Intervention	52	2 g/day PUFA (EPA:DHA 1.2:1)	Before CABG until discharge	64	77
				Placebo	51	2 g/day olive oil		68	82
Sandesara *et al* 2010	USA	R, DB, PC	Post-CABG, SR	Intervention	120	4 g/day PUFA before CABG, 2 g/day PUFA after CABG (EPA:DHA 1.24:1)	Preoperatively until primary endpoint or 14 days	63	78
				Placebo	123	4 g/day corn oil before CABG, 2 g/day corn oil after CABG		62	83
Bianconi *et al* 2010[25]	Italy	R, DB, PC	Persistent AF >1 month Post-cardioversion	Intervention	95	3 g/day PUFA 1 week before and 2 g/day thereafter (EPA:DHA 1.2:1)	1 Week before and 6 months after EC	69	67
				Placebo	92	Placebo		69	62
Nodari *et al* 2010*	Italy	R, PC	Persistent AF >1 month and on amiodarone and RASi; Post-cardioversion	Intervention	100	1 g/day PUFA	NA	70	NA
				Placebo	99	Placebo		69	
Kowey *et al* 2010	USA	R, DB, PC	Symptomatic paroxysmal and persistent AF (5:1)	Intervention	322	4 g/day PUFA with loading dose of 8 g/day for the first 7 days (EPA:DHA 1.24:1)	24 Weeks after enrolment	60	60
				Placebo	323	4 g/day corn oil with loading dose of 8 g/day for the first 7 days		61	53

Figure 4.1: Cumulative effect of n-3 PUFA therapy on incidence of AF following CABG surgery (from Liu et al)



The second clinical setting chosen by the researchers conducting the meta-analysis (Liu T et al) was recurrence of AF following reversal to sinus rhythm with external DC cardioversion in patients with persistent AF. In this analysis, the researchers evaluated the cumulative outcome data from 5 clinical stidues [142-146] and again reported that n-3 PUFA did not reduce the recurrence of AF following cardioversion as shown in figure 4.2.

Figure 4.2: Effect of n-3 PUFA on the risk of recurrence of AF (from Liu et al)



4.1.3: Monitoring for AF episodes in clinical studies

An important variable that would have a significant impact on the reported outcome in all these studies is the method of arrhythmia monitoring and as discussed in detail above, we addressed this issue in a robust fashion in our study. The analysis by Liu et al again addressed this question and concluded that there is significant variability in the methods used for arrhythmia monitoring within the studies included in this analysis and consequently the reported incidence of AF in the control population is very variable as shown in table 37.

Table 37: Method of monitoring for AF in studies on the effect of n-3 PUFA on AF (Liu et al)

Author (study) year	Follow-up period	Definition of AF	Methods of AF detection	Rate of AF in control group
Calo *et al* 2005	During hospitalisation	Postoperative inhospital AF >5 min or requiring intervention	Continuous monitoring at least 4–5 days after the operation; ECG daily until discharge; AF endpoint adjudicated by a blinded external committee	33.3%
Erdogan *et al* 2007	52 Weeks	Recurrent AF	Clinical examination with ECG after 4 weeks, 12 weeks and 1 year or at any point of occurrence of symptoms	85.2%
Margos *et al* 2007	6 Months	Recurrent AF	24 h Holter at 1 month, ECG at 1, 3 and 6 months	40%
Heidt *et al* 2009	During ICU	Postoperative inhospital AF ≥15 min	Continuous monitoring and ECG daily until transfer from the ICU to a normal ward	30%
Heidarsdottir *et al* 2010	During hospitalisation	Postoperative inhospital AF ≥5 min	Continuous monitoring until discharge	54.1%
Saravanan *et al* 2010	5 Days	Postoperative inhospital AF ≥30 s	Continuous monitoring at least 5 days after the operation; then ECG daily until discharge; AF endpoint adjudicated by a blinded external committee	43.1%
Sandesara *et al* 2010	14 Days	Postoperative clinically significant AF or atrial flutter documented by rhythm strip or ECG and requiring treatment	Continuous monitoring and ECG; AF endpoint adjudicated by a blinded external committee	33%
Bianconi *et al* 2010	6 Months	Recurrent AF	Trans-telephonic monitoring three times during first week and then twice a week until the 3 month follow-up and ECG at 48–72 h, 1 week and 1, 3 and 6 months AF endpoint adjudicated by the blinded investigators	51.1%
Nodari *et al* 2010	1 Year	Recurrent AF	ECG and 24-h Holter at 1, 3, 6 and 12 months	63%
Kowey *et al* 2010	6 Months	Recurrent AF	Trans-telephonic monitoring every 2 weeks; AF endpoint adjudicated by the blinded investigators	46%

4.1.4: Conclusion on the effect of n-3 PUFA in AF following CABG

Thus our conclusion that n-3 PUFA supplementation pre-operatively does not reduce the risk of AF following CABG is well validated. In addition, there appears to be no benefit with n-3 PUFA supplementation on the risk of recurrence of AF following cardioversion. However, as epidemiological and observational studies [80,147] have shown a reduced incidence of new onset AF in individuals with high dietary n-3 PUFA consumption over a long period of time, it is relevant to see if longer duration of supplementation in patients "at risk" of AF would reduce their chance of developing AF. This question is particularly important as one recent report suggests that n-3 PUFA supplementation slows the rate of re-modelling of atrial substrate in a heart failure animal model and reduces the risk of new onset AF [148]. Thus there is still some benefit to be achieved in evaluating the role of n-3 PUFA in various other clinical settings of AF as n-3 PUFA could be a safe alternative to anti-arrhythmic drug therapy with their attendant risk of significant side effects on long term administration, in these sub-sets of patient population.

4.1.5: Is Post operative AF a "Good Model" to study AF?

Atrial fibrillation is a heterogeneous disease that affects various age groups. Often young patients (≤35 years) have lone atrial fibrillation in the absence of structural heart disease, whereas older individuals (typically ≥65 years) have underlying cardiovascular disorders that result in structural remodelling of the atrium, predisposing to atrial fibrillation. It is also the most common cardiac arrhythmia reported in clinical practice. Drug treatments for this disorder are restricted by pro-arrhythmic effects on the ventricles, and a need exists to identify effective drugs that can be used to treat atrial arrhythmias with a minimum risk of ventricular arrhythmia. Therefore, the effect of n-3 PUFA on this very common arrhythmia deserves further evaluation. The negative finding in our study and

several subsequent studies clearly demonstrates the futility of this intervention to prevent AF following CABG. However, the pathogenesis of AF following CABG is likely to be considerably different from the common forms of AF seen in clinical practice as there are factors which are unique to this setting such as

1) Immune and inflammatory activation due to the surgery
2) Atrial ischaemia during bypass
3) Oxidative reperfusion injury
4) A new scar in the atrium (as a result of atriotomy to connect to the by-pass circuit)
5) Perturbations of autonomic tone
6) Concomitant use of adrenergic agents (inotropes)

that would not be seen to play a role in the initiation and sustenance of other forms of AF. This in addition to findings of a prospective population-based study of adults 65 years, where the investigators reported that risk of new onset AF was considerably lowered with consumption of grilled or baked fish (147), with a possible dose-response effect that was confirmed by measurements of plasma concentrations of eicosapentaenoic acid and docosahexaenoic acid and another observational study in men older than 42 years (149), which reported that high serum concentrations of docosahexaenoic acid had a protective effect against atrial fibrillation during a follow-up of 17 years raised the possibility that n-3 PUFA may have a beneficial effect in other forms of AF particularly when administered over longer periods of time.

4.1.6: Does n-3 PUFA increase the risk of AF? (Pro-arrhythmic properties of n-3 PUFA)

An interesting observation in our study was that patients who received n-3 PUFA had a slightly higher incidence of AF episodes. Even though the effect was not statistically

significant and could well be a chance association, we could not confidently rule out this possibility as this study was powered to look for a beneficial effect of the magnitude shown in the previous study by Calo et al. In order for us to have been able to confidently rule out a pro-arrhythmic effect (harm from treatment), given the magnitude of the change seen in our study, we would have needed a much larger sample size (224 in each group). The likelihood of n-3 PUFA exerting a pro-arrhythmic effect in some clinical situations (stable angina, ICD recipients) has previously been reported. The cellular basis for such an effect has been elaborately reviewed by Den Ruijter et al [(51)].

4.1.7: AF, Inflammation and n-3 PUFA as an anti-inflammatory agent

The peak incidence of AF after cardiac surgery coincides with the peak concentration of inflammatory indices [(150)] and it is well known that the incidence of AF is high in a setting of systemic inflammation such as systemic inflammatory response syndrome (SIRS) or organ based inflammatory conditions such as pericarditis and pneumonia. In keeping with this, statins, drugs with known anti-inflammatory properties, have been consistently reported to exhibit some anti-arrhythmic effect in the setting of postoperative AF [(151)]. There is evidence suggesting that fish or n-3 PUFA consumption attenuates inflammation and oxidative stress in humans [(152, 153)]. Thus there is a potential for n-3 PUFA to have suppressed inflammation and reduced the incidence of AF in the study by Calo et al. To specifically address this issue, we estimated the levels of highly sensitive C-reactive protein (HS-CRP) as a marker of systemic inflammation in our study group. We performed estimation of HS-CRP at base line and after 48 hours of surgery (the usual time when patients experience peak inflammatory response) in both groups of patients and failed to see a significant difference with therapy with n-3 PUFA. This could be because the dose used in our study (2g/day) was not sufficient to suppress inflammation as most

studies where a significant anti-inflammatory response is seen have used much larger doses. Notwithstanding this fact, we could conclude that the beneficial effects reported by Calo et al could not have been due to suppression of inflammatory response.

4.1.8: AF, Autonomic regulation and effect of n-3 PUFA on autonomic function

The influence of changes in the autonomic nervous system in the initiation and maintenance of paroxysmal AF is well documented [154,155]. Electrophysiological characteristics of atrial cells (action potential duration and refractoriness, conduction speed) are modulated variably by para-sympathetic (vagal) and sympathetic influences [156]. The former tends to favour macro-reentry phenomena whereas the latter favours abnormal automaticity and triggered activity. In normal hearts vagal influences are predominant. A common clinical pattern of vagal-mediated paroxysmal atrial fibrillation is frequently observed in young, fit adults in the absence of structural heart disease. Sympathetically mediated atrial fibrillation is often observed in the presence of structural heart disease and common clinical conditions associated with systemic inflammation such a pneumonia and pericarditis. The initial effect that is pro-arrhythmic in such situations with high sympathetic drive is provocation of a "vagal withdrawal". Thus, the clinical situation in which AF is observed indicates one or the other mechanism to predominate as the cause of AF. Often observing the behavior of sinus rate variability (heart rate variability-HRV) just preceding the onset of the arrhythmia may permit evaluation of the mechanism of arrhythmia. Following cardiac surgery, a heightened sympathetic activation and vagal withdrawal are both seen which could contribute to the high incidence of AF in this setting [157].

Dietary intake of n-3 PUFA have been shown to alter autonomic tone in humans [158] with higher n-3 PUFA levels associated with greater para-sympathetic (vagal) tone and a slower resting heart rate [160]. Several studies have reported that higher dietary intake of n-3 PUFA has a significant and consistent influence on the markers of autonomic tone such as heart rate variability and other indices of autonomic function with the balance being shifted in favor of a cholinergic excess [161-163]. This effect could have a significant anti-arrhythmic potential particularly in relation to risk of developing ventricular arrhythmias and sudden cardiac death. The mechanism of such beneficial effect is unclear. None of the studies that showed a beneficial modulation of the autonomic tone evaluated the levels of n-3 PUFA in serum and/ or memebrane incorporation. As our study design included estimation of all these levels following short term supplementaiton, we estimated the influence of n-3 PUFA on various markers of autonomic function and compared that with changes at the level of the receptors that mediate the cardiac autonomic activity.

In our study, even though n-3 PUFA supplementation did not reduce the incidence of atrial fibrillation following cardiac surgery, we were able to replicate the findings in most other clinical situations where n-3 PUFA supplementation had led to a favorable modulation of autonomic function with a predominance of cholinergic tone. This was evidenced by a higher high frequency signal (a marker of cholinergic dominance) on the heart rate variability. Interestingly, we noticed a significant alteration in the expression level of cardiac M2 autonomic receptors in the n-3PUFA treated group which might sugest a possible mechanism of such beneficial effect as detailed below.

4.1.9: Effect of n-3 PUFA on expression of m-RNA levels of cardiac autonomic receptors

The mechanism by which n-3 PUFA modulate autonomic tone is unclear. The influence could be either central within the nervous system or at the receptor level. Considerable evidence exists that a change in the lipid composition of biological membranes is closely associated with alterations in their function, and in almost all studies with dietary supplementation with n-3 PUFAs, an increase in phospholipid EPA and DHA in cell membranes has been observed. Often, these alterations in the fatty acid composition leads to changes in the expression or affinity of various membrane bound receptor proteins. In addition, as discussed above, n-3 PUFA incorporation into cell membranes can lead to changes in the membrane fluidity [(56)], which may in turn alter the dynamics of membrane bound enzyme and receptor functions.

We hypothesised that the beneficial effects of n-3 PUFA seen on the autonomic nervous system is likely to be due to a change at the receptor level rather than a central effect on the nervous system. We performed qPCR assay for the common cardiac autonomic receptors namely β1, β2 and M2 receptors. This showed that n-3 PUFA supplementation and subsequent membrane incorporation was associated with a greater expression of m-RNA of M-2 muscarinic receptors without a significant change in the expression levels of beta-adrenergic receptors. Thus, it is possible that the increased cholinergic response observed in many studies and replicated in our study could at least partly be due to a higher expression levels of M2 receptors such that for a given cholinergic output there is a greater cholinergic response at the end organ level.

4.1.10: ECG markers of risk of developing AF and the effect of n-3 PUFA on these markers:

P-wave on a surface 12 lead ECG tracing is a reflection of atrial electrical activation. Alterations in the properties of P-waves such as maximum duration of P wave (P-max), P wave dispersion (PWD-difference between the maximum duration and the minimum duration within the 12 leads) have been reported to predict risk of developing new onset atrial fibrillation [(164)]. A recent report by Magnani et al [(165)] on P-wave duration and risk for longitudinal atrial fibrillation from the Framingham Heart Study, using a single-channel electrocardiographic (ECG) recording, found that maximum P-wave duration at the upper fifth percentile was associated with long-term AF risk.

Buxton and Josephson [(166)] reported that simultaneous recording of ECG leads enables calculation of "P-wave dispersion index" -a method of assessing heterogeneity in conduction of electrical impulses within regions of the atrium. P-wave dispersion has been reported to be a simple ECG predictor of paroxysmal lone AF [(164)] Although acceptable intra-observer and inter-observer error in the measurement of P-wave duration on 12-lead electrocardiography have been reported, [(164)] well-known difficulties in defining P-wave onset and offset may restrict the accuracy and reproducibility of the measurements. To overcome these restrictions magnified "callipers" on standard computer screens have proved useful in the accurate evaluation of common P-wave descriptors [(167)].

The effect of n-3 PUFA supplementation on P wave parameters has not been reported so far. In this study, we evaluated the P wave parameters in a 12 lead ECG pre and post supplementation and compared this with the outcome of atrial fibrillation. We showed that short term n-3 PUFA supplementation (with evidence of membrane incorporation) did not alter any of the P-wave parameters. This was consistent with our

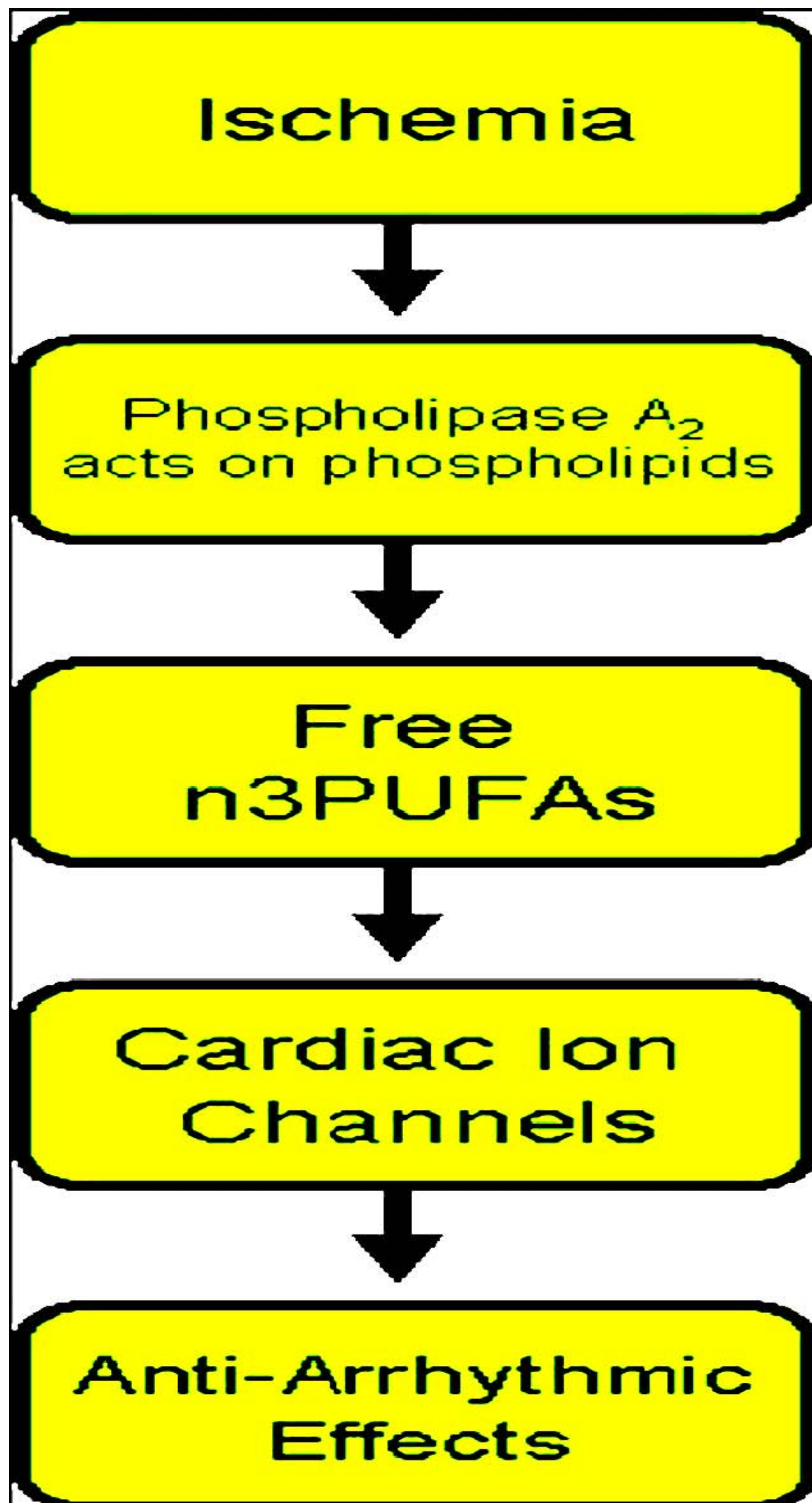
main finding that n-3 PUFA did not reduce the incidence of atrial fibrillation. One possible limitation in this aspect of the study could be the fact that there are several factors which influence the P wave indices within a given patient (diurnal variation, changes with activity levels) [(168)]. However, as all ECGs were done at rest at roughly similar time of the day and the strength of randomisation that is inherent in the study design would have compensated for this potential confounding influence. Thus, we showed that n-3 PUFA supplementation did not have a significant influence on this ECG parameter of atrial arrhythmogenesis.

4.1.11: Free and incorporated n-3 PUFA levels and their effect on arrhythmias.

The complex interaction between circulating and tissue levels of n-3 PUFA and the resulting electrophysiological alterations are not completely understood and may partly explain the variability in results across clinical trials [(140,169,170,171)]. Some effects of n-3 PUFA that are seen on acute application are not seen after incorporation- particualrly the inhibitory effect on sodium channels which is very pronounced in acute application. Interestingly, Metcalf et al [(172)] demonstrated that fish oil supplementation in humans causes a gradual increase in tissue levels of n-3 PUFA over few weeks to 2-months, whereas plasma levels rise abruptly and remain constant thereafter. Therefore, it is possible that the very short term (average of 5 days) administration of n-3 PUFA in the study by Calo et al and the Intravenous administration in the study by be Heidt et al could have increased the serum levels but not resulted in tissue incorporation. Such increase in srum levels without tissue incorportation could mimic acute application in the electrophysiological laboratory and may have a more pronounced anti-arrhythmic effect. Moreover, there is increasing evidence that local relase of n-3 PUFA from the membrane

incorporated phospholipid is necessary for its anti-arrhythmic action and that this effect may be dependant on the activity of phospholipase A2 as shown in figure 4.3.

Figure 4.3: A flow diagram of the proposed protective effect of n-3 PUFA during ischemia.



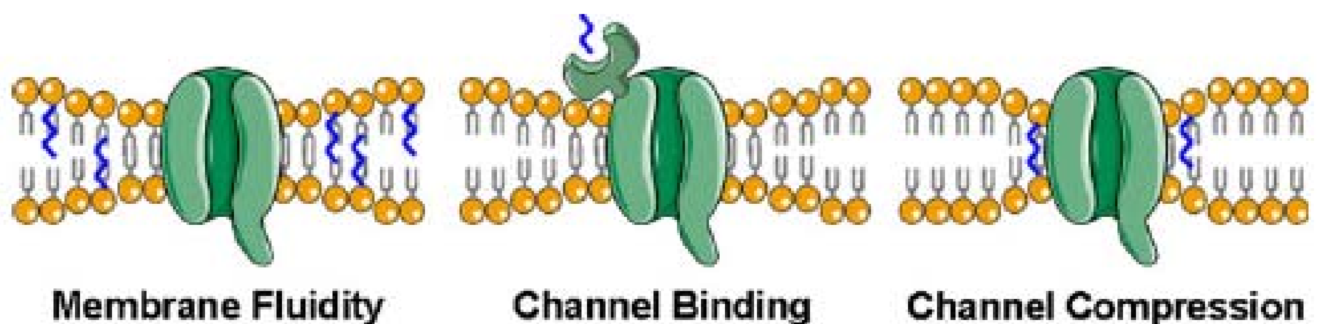
Many studies have reported increased EPA and DHA in plasma or serum lipids following supplementation of the diet with n-3 PUFA [173]. The increment in these fatty acids seen in the current study in serum phospholipids (86% increase in EPA and 45% increase in DHA) is consistent with existing data. However, the current study also shows higher EPA and DHA in phospholipids of PBMC and atrial tissue after supplementation with n-3 PUFA for a relatively short period of time. The higher EPA and DHA content of atrial phospholipids after n-3 PUFA supplementation observed in the current study supports the observation of Metcalfe et al [172] that these fatty acids, even with short term supplementation, are readily incorporated into this tissue.

In animal experiments, it has been shown that the electrophysiological effects of fish oils applied to ventricular myocytes in suspension vary significantly from those of the membrane-incorporated n-3 PUFA [51] and this has been reflected by a variable effect of acute (often intravenous) vs. chronic (dietary supplementation) administration of n-3 PUFA on cardiac arrhythmias in man [170]. In a clinical setting this would have a bearing on the dose, duration and method of administration of n-3 PUFA and the consequent variation in circulating and tissue levels of n-3 PUFA. The complex interaction between circulating and tissue levels of these fatty acids, the effect of stress on free fatty acid release from adipocytes, and the resulting changes in the electrophysiology of cardiomyocytes are not well understood and may explain much of the diversity in the outcome of clinical studies. In the current study, both the serum and tissue levels of n-3 PUFA were measured. To our knowledge, this is the first time that tissue levels of n-3 PUFA have been measured in a clinical study evaluating the anti-arrhythmic potential of these fatty acids.

4.2: n-3 PUFA and expression level of ion channels, transporters and calcium handling proteins

There is considerable volume of evidence to show that n-3 PUFA influence the effect of various cardiac ion channel function in isolated cardiomyocytes both upon acute application and incorporation (by dietary supplementation) [51]. The effects seem to vary with the nature of n-3 PUFA exposure (acute application VS dietary supplementation) and there also appears to be a significant inter-species variability. Moreover, the multitude of effects makes it difficult to attribute the beneficial anti-arrhythmic property to a specific mechanism of action. In addition, n-3 PUFA supplementation changes membrane phospholipids composition significantly and the overall fluidity of the membrane seems to alter. Currently there are three plausible mechanisms that have been postulated to explain this diverse functional influence on ion channel activity by n-3 PUFA. To date, three main hypotheses attempt to explain how n-3 PUFA may exert their influence on cardiac ion channels and thus, the electrophysiology of the heart as shown in figure 4.4 [174].

Figure 4.4: Possible mechanisms of action of n-3 PUFA on ion channels (from Xiao et al)



First, those ion-channels modified by n-3 PUFA may contain a specific binding site for n-3 PUFA that, if occupied, can alter their function. This is supported by evidence from Xiao et al. [175], who showed that a single-point mutation in the alpha subunit of the Na+ channel prevented EPA from inhibiting channel currents. This group also showed that co-expression of the alpha subunits with the beta subunits significantly altered the ability of n3 PUFA to affect the channels [176]. The second hypothesis is that n-3 PUFA may be affecting membrane fluidity and, subsequently, ion channel function. For example, Jahangiri et al.[177] correlated the effect of n-3 PUFA on ion channels to changes in membrane fluidity by monitoring steady-state fluorescence anisotropy. This hypothesis may also explain why n-3 PUFA have such widespread effects on membrane-dwelling channels and pumps. However, some argue that the observed effects on ion channels occur at much lower concentrations than those necessary to change membrane fluidity. Also, the lack of significant effects of n-3 PUFAs on inward rectifier K+ channels (IK1) and human hyperpolarization-activated, cyclic nucleotide-gated (hHCN4) channels [178] is not along the line of this hypothesis. The third main hypothesis considers that changes in the membrane immediately surrounding the channels might be responsible for the ion channel effects. The hydrophobic lengths of ion channels are often less than the hydrophobic thicknesses of membrane phospholipids, so the membrane must “pinch” together around the ion channels. This compression causes tension on the channels and affects their function [179]. It is considered that n-3 PUFA may insert in the membrane around the channels which, in turn, relieves some of the tension by reducing the curvature of the membrane. It has been shown that detergents which may relieve tension on ion channels induce similar effects to n-3 PUFA [180]. However, none of these mechanisms are able to explain the overall effect on the ion channels. We therefore hypothesized that n-3 PUFA may act by altering expression levels of various ion channels, transporters and/ or

calcium handling proteins which could then result in the observed functional effect rather than a direct inhibitory or excitatory effect. Even though, evidence in relation to alteration in the expression levels of these proteins is lacking (which makes this study interesting and relevant), there is some evidence from animal studies to suggest that in dogs fed n-3 PUFA, the expression levels of cardiac connexins (one of the inter-cellular communication channels) is altered significantly. In view of the multitude of functional effects seen in animal experiments involving several ion channels, transporters and calcium handling proteins, we performed quantitative PCR as a mass screening investigation to evaluate for changes in mRNA levels of a number of relevant proteins which are potential candidates that could be altered to effect a change in the cardiac arrhythmogenesis.

4.2.1: qPCR- mRNA levels of cardiac ion channel proteins

Using quantitative PCR, we evaluated a number of targets which included sodium channels, potassium channels and calcium channels and this revealed that there was no significant change in the expression of any of the cardiac ion channel proteins that have previously been reported to be showing functional alterations [51,174]. This may be due to the short duration of supplementation thus not allowing enough time for such alterations to be manifest. Of note, most animal studies (Mouse, rats and pigs) that showed a functional change upon incorporation used supplementation for periods ranging from 4-16 weeks, which in a human context could be expected to be much longer. However, in our study, we clearly demonstrated a change in the constitution of the membrane phospholipids. If the main mechanism of n-3 PUFA on ion channels was indeed mediated by an alteration in the expression level of membrane bound ion channel proteins, we would have expected to see some difference in the m-RNA levels of those ion channels

where animal experiments have previously reported a significant functional alteration. In addition, the fact that acutely applied n-3 PUFA could alter functional properties of ion channels indicate that n-3 PUFA should be able to exert a direct effect on the ion channels themselves rather than altering their membrane expression.

4.2.2: Effect of n-3 PUFA on expression of m-RNA of cardiac connexins

Gap junctions form the intercellular pathway for cell-to-cell transmission of the cardiac impulse from its site of origin, the sinoatrial node, along the atria, the atrioventricular conduction system to the ventricular myocardium. The component parts of gap junctions are proteins called connexins (Cx), of which three main isoforms are found in the conductive and working myocardial cells: Cx40, Cx43, and Cx45. These isoforms are regionally expressed in the heart, which suggests a specific role or function of a specific connexin in a certain part of the heart. Cx40 is mainly expressed in the atrial myocytes, in the AV-node, His-bundle and the ventricular conduction system while Cx43 is by far the most abundant and is expressed between atrial and ventricular myocytes and distal parts of the conduction system. Cx45 is mostly seen within the cardiac conduction tissue [(181)]. The ratio between Cx40 and Cx43 seems to alter conduction properties and has been reported to influence the risk of initiation and maintenance of atrial arrhythmias [(182)]. One study on dogs with vagal mediated AF reported that n-3 PUFA supplementation altered expression of connexin in the dog atria and reduced the risk of vagal mediated AF [(183)]. In our study, there was no significant change in the expression levels of cardiac connexins in the human right atrium following incorporation of n-3 PUFA in membrane phospholipids. Whilst this would suggest that n-3 PUFA may not have an important effect on the cardiac connexins, it is important to note that there is considerable variation in the regional distribution of connexins and we estimated connexin levels in right atrial appendage. The

absence of a significant alteration at the RAA does not preclude a change that could have manifest in other regions of the atria. Of note, much of the risk of atrial fibrillation is linked to alteration in conduction velocities within the left atrium. In addition, the short duration of supplementation is also another important factor to be kept in mind while deriving conclusions from our finding. Notwithstanding these caveats, our findings suggest that short term n-3 PUFA supplementation does not alter the expression levels of cardiac connexins in human right atrial appendage.

4.2.3: Effect of n-3 PUFA on expression levels of calcium handling proteins

In our study, we hypothesized that n-3 PUFA supplementation would have a significant effect on the expression level of the cardiac calcium handling proteins which could explain some of the effects seen above and may translate as a beneficial influence on the incidence of AF following CABG. We also sought to evaluate the functional effect on cardiac calcium handling in human atrial myocytes after supplementation with n-3 PUFA however, due to technical difficulties as described previously, we were unable to do so. Hence we estimated the effect of incorporation of n-3 PUFA in rat ventricular myocytes to supplement the data obtained in relation to the expression levels of cardiac calcium handling proteins.

qPCR for cardiac calcium handling proteins showed a significant reduction in the expression of total phospholamban without a concomitant change in the expression of SERCA. Our data also showed a significant increase in the expression level of RYR2 receptors. There was no change in the expression levels of NCX or SERCA. A similar decrease in the expression level of phospholamban and an increase in the expression of

RYR2 were shown by using semi-quantitative analysis of fluorescent signals during Immunohistochemistry on tissue sections in these patients.

Western blot analysis to quantify the target proteins involved in cardiac calcium handling showed a significant decrease in the quantity of phospholamban however, the increase seen in the expression of m-RNA of RYR2 in the qPCR and in the tissue sections by Immunohistochemistry was not seen in Western blot analysis. The reason for this lack of a significant change in the protein level of RYR2 receptor in the Western blot technique while the other two methods suggest a possible increase in the expression could be that the sensitivity of the Western blot technique is not high enough to detect a change that might be significant. Alternatively, it may also be possible that the change seen in the message (as m-RNA) had not translated into a protein level change as the duration of supplementation in our cohort was short.

Notwithstanding this limitation in our data, we could conclude that n-3 PUFA supplementation reliably reduced the expression level of cardiac phospholamban in human right atrial appendage while it is likely to have had an effect that would translate as increased expression of RYR2 receptors.

4.2.4: Alteration in the expression of cardiac phospholamban

Phospholamban is the major protein that regulates (inhibits) SR calcium ATPase (SERCA) activity. The observed reduction in the expression level of cardiac phospholamban in the absence of a concomitant change in the SERCA is likely to increase the activity of SERCA. This might translate as increased calcium re-uptake into the SR and may increase the SR calcium content. As diastolic calcium release is predominantly dependant on the cytosolic calcium level, a more effective (or more rapid)

re-uptake of cytosolic calcium into the storage site (SR) could suppress diastolic calcium release which could be anti-arrhythmic. An important caveat to this discussion relates to phosphorylation of phospholamban, which relieves the inhibitory effect of phospholamban on SERCA. Unfortunately, within the remit of this study, we were unable to measure the phosphorylated phospholamban levels due to lack of time and adequate resources. However, in our group, we have previously shown that in rat ventricular myocytes perfused with EPA, the rate of phosphorylation of phospholamban was significantly increased [194] (4 fold increase). Thus, the only available data on the effect of n-3 PUFA on phosphorylation of phospholamban indicates that there is likely to be an increase rather than a decrease in the rate of phosphorylation. Hence, the observed effect of reduced expression of total phospholamban would lead to a considerable increase in SERCA activity if it were to be associated with a concomitant increase in its rate of phosphorylation. A concomitant increase in the expression level of RYR2, assuming that the expression translates as increased number of "open" RYR2 available (which has clearly not been shown in this study as the study by its nature was not designed to test the open probability of RYR2) then one could expect a more effective release of calcium from the SR and thus a more efficient calcium cycling mechanism. On the contrary, a more rapid re-uptake of cytosolic calcium into the SR could lead to SR calcium overload that might exceed the threshold for calcium release and could lead to spontaneous release of calcium which could be pro-arrhythmic. It is also possible that the observed reduction in expression of phospholamban and an increase in the expression level of RYR2 could be a compensatory phenomenon to direct inhibition of SERCA and RYR2 respectively, which might explain an interesting observation that n-3 PUFA application reduces systolic and diastolic calcium transients in heart failure without adversely affecting contractility. In summary, the observed changes in the expression level of cardiac

phospholamban and RYR2 could have either an anti-arrhythmic or pro-arrhythmic effect or it could simply be a compensatory phenomenon and further work is clearly indicated to elucidate the functional significance of this finding.

4.3: Effect of n-3 PUFA on calcium handling in rat ventricular myocytes

In order to correlate the data on the expression level of calcium handling proteins to their functional relevance, we used rat ventricular myocytes obtained from rats supplemented with n-3 PUFA enriched diet loaded with calcium sensitive dye Fluo-3 and evaluated the systolic calcium transient and the response to application of caffeine.

In our study, we showed that the amplitude of the systolic calcium transient in n-3 PUFA supplemented rat ventricular myocytes was not significantly different from that of the control cells. This is in contradiction to the data from the study by Den Ruijter et al where they reported a significant (>25%) reduction in amplitude of the systolic calcium transient upon acute application. However, we showed a much rapid decay of the systolic calcium transient in the n-3 PUFA supplemented cells which will co-relate well with increased SERCA activity as the function of SERCA is to promote re-uptake of calcium from the cytosol into the SR. Upon application of caffeine, the SR empties its entire contents into the cytosol and a measure of the amplitude of the calcium transient obtained upon application of caffeine is an indirect measure of SR calcium content. Even though there are several factors that would need to be considered, not the least, the action potential duration, which would have an important effect on the SR calcium content, and we did not measure the action potential in our study, we did show that the amplitude of the calcium transient obtained after application of caffeine was significantly higher in the n-3 PUFA treated cells. This could again indicate a greater activity of the SERCA promoting

re-uptake of calcium from the cytosol leading to a higher SR calcium content. Interestingly, the rate of decay of the caffeine transient was not significantly different thereby suggesting that the faster rate of decay of the systolic calcium transient observed could indeed be due to greater SERCA activity as SERCA is completely blocked by application of caffeine. The other important variable that has not been measured is the L-type calcium current which would have a bearing on our interpretation of our results.

In summary, the field stimulation experiments that we carried out shows that n-3 PUFA incorporation into rat ventricular myocytes does not alter the amplitude of the systolic calcium transient but accelerates the decay of the transient. It also increases the amplitude of the caffeine transient but does not alter the rate of decay all of which could be due to greater SERCA activity which would be consistent with a decreased expression level of phospholamban that was shown in all the three modalities of molecular biological techniques.

4.4: Summary of findings

1) N-3 PUFA supplementation in the immediate pre-operative period does not reduce the risk of AF following CABG surgery.

2) N-3 PUFA supplementation does not have any effect on the ECG markers of atrial arrhythmogenesis

3) In humans, N-3 PUFA supplementation appears to have a beneficial modulation of the cardiac autonomic response with a higher vagal response. This is associated with an increased expression level of cardiac M2 receptors in the right atrial appendage of patients supplemented with n-3 PUFA. Such increase in expression could lead to a situation where a given vagal neural stimulus could produce a greater cholinergic response.

4) N-3 PUFA supplementation reduces the expression level of cardiac phospholamban and increases the expression level of cardiac ryanodine receptor in human right atrial appendage.

5) In rat ventricular myocytes, N-3 PUFA supplementation does not alter the systolic calcium transient but increases the rate decay of the systolic calcium transient and increases the amplitude of the caffeine transient thus indicating a greater re-uptake of calcium into the SR and a higher SR calcium content both of which could be mediated by a greater activity of SERCA which could be due to lower expression of cardiac phospholamban (the inhibitory regulator of SERCA).

4.5: Study Limitations

1) Lack of demonstrable change in the RYR protein by Western Blot

2) Functional relevance of changes in expression not clearly elucidated

3) Species difference between human atrium (expression levels) and rat ventricle (calcium cycling)

4) Lack of data on the expression levels in rat ventricular myocytes.

4.6: Future directions:

4.6.1: Clinical :

It is clear that n-3 PUFA supplementation does not reduce the risk of AF following CABG, however, the role of long term n-3 PUFA supplementation in the primary prevention of new onset AF in patients at risk of AF (patients with conditions that are known to predispose to AF such as hypertension, heart failure, mitral valve disease etc) is not clear and a large clinical trial with a long duration of follow-up will be able to address this important and interesting research question as there is an urgent need for a safe and effective drug that would prevent AF.

4.6.2: n-3 PUFA, cardiac calcium regulation and AF

This is the first study to evaluate the expression levels of cardiac ion channels, transporters and calcium handling proteins in humans after incorporation of n-3 PUFA. The major mechanism of action of n-3 PUFA is still not clear. The findings of this study,

whilst generate interesting hypothesis, are not sufficient to address this question effectively. It is interesting to note that a short duration of supplementation has indeed changed the expression levels of cardiac calcium handling proteins whilst having no significant effect on the ion channel expression, this could indicate that much of the anti-arrhytmic benefits seen in clinical studies following a myocardial infarction and heart failure (2 conditions where arrhythmias are precipitated by cellular calcium overload) could be due its beneficial influence on cardiac calcium handling. This question is indeed very important and can be addressed by a further study evaluating the effect of incorporated n-3 PUFA on the calcium handling properties of human ventricular myocytes obtained during heart transplant surgery (explanted hearts). This could be achieved by selecting patients who are in the elective list for heart transplant and supplementing them with oral n-3 PUFA for a period prior to transplant surgery.

References

1. Gudbjarnason J, Oskarsdottir S, Doell G, Hallorimsson B. J. Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardio125, 130-144 (1978)].

2. Rocquelin G, Guenot L, Justrabo E, Grynberg A, David M. Fatty acid composition of human heart phospholipids: data from 53 biopsy specimens. J Mol Cell Cardiol. 1985 Aug;17(8):769-73

3. Rocquelin G, Guenot L, Astorg PO, David M. Phospholipid content and fatty acid composition of human heart. Lipids. 1989 Sep;24(9):775-80.

4. Mandy L. Theiss ML, McLennan PL, Sheeran F, Pepe S. Fatty acid composition of human heart: Setting the baseline. J Mol Cell Cardiol Volume 42, Issue 6, Supplement 1, June 2007, S116-S117.

5. Simopoulos AP, Leaf A, Salem N Jr. Workshop statement on the essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids. 2000 Sep;63(3):119-21.

6. Tribole, E.F.; Thompson, RL; Harrison, RA; Summerbell, CD; Ness, AR; Moore, HJ; Worthington, HV; Durrington, PN. "Excess Omega-6 Fats Thwart Health Benefits from Omega-3 Fats". BMJ 332 (7544): 752–760.

7. Lands WE. Dietary fat and health: the evidence and the politics of prevention: careful use of dietary fats can improve life and prevent disease. Ann N Y Acad Sci. 2005 Dec;1055:179-92.

8. Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. Am J Clin Nutr. 2006 Jun;83(6 Suppl):1483S-1493S.

9. Falk-Petersen, S., Sargent J.R., Henderson, J., Hegseth, E.N, Hop, H. and Okolotkov, J.B. 1998. Lipid class and fatty acid compositions of ice-algae and phytoplankton from the Marginal Ice Zone, the Barents Sea. Polar Biol. 20: 41-47.

10. "Fish, Levels of Mercury and Omega-3 Fatty Acids". American Heart Association. Recommendation- Fish 101.

11. Harris WS. Fish oil supplementation: evidence for health benefits. Cleve Clin J Med 2004; **71:** 208–10.

12. Wood DA, Kotseva K, Connolly S, et al, on behalf of the EUROACTION Study Group. Nurse-coordinated multidisciplinary, family-based cardiovascular disease prevention programme (EUROACTION) for patients with coronary heart disease and asymptomatic individuals at high risk of cardiovascular disease: a paired, cluster-randomised controlled trial. Lancet 2008; **371:** 1999–2012.

13. Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med 2004; **39:** 212–20.

14. Lloyd-Jones DM, Wang TJ, Leip EP, Larson MG, Levy D, Vasan RS, D'Agostino RB, Massaro JM, Beiser A, Wolf PA, Benjamin EJ. Lifetime Risk for Development of Atrial Fibrillation: The Framingham Heart Study Circulation, Aug 2004; 110: 1042 - 1046.

15. Furberg CD, Psaty BM, Manolio TA, Gardin JM, Smith VE, Rautaharju PM. Prevalence of atrial fibrillation in elderly subjects (the Cardiovascular Health Study). Am J Cardiol. 1994 Aug 1;74(3):236-41.

16. Kannel WB, Wolf PA, Benjamin EJ, Levy D. Prevalence, incidence, prognosis, and predisposing conditions for atrial fibrillation: population-based estimates. Am J Cardiol. 1998 Oct 16;82(8A):2N-9N

17. Benjamin EJ, Wolf PA, D'Agostino RB, Silbershatz H, Kannel WB, Levy D. Impact of Atrial Fibrillation on the Risk of Death - The Framingham Heart Study. Circulation. 1998;98:946-952.

18. Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation: a major contributor to stroke in the elderly: the Framingham Study. Arch Intern Med.. 1987;147:1561-1564. [Abstract]

19. Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. Stroke. 1991;22:983-988.

20. Dialy B, Lehmann MH, Schumacher DN, Steinman RT, Meissner MD. Hosptalization for arrythmias in the united states: Importance of atrial fibrillation. J Am Coll Cardiol 1992;19:41A

21. Mathew JP, Parks R, Savino JS, Friedman AS, Koch C, Mangano DT, et al. Atrial fibrillation following coronary artery bypass graft surgery: predictors, outcomes, and resource utilization. MultiCenter Study of Perioperative Ischemia Research Group. JAMA. 1996;276:300-6.

22. Andrews TC, Reimold SC, Berlin JA, Antman EM. Prevention of supraventricular arrhythmias after coronary artery bypass surgery. A meta-analysis of ran-domized control trials. Circulation. 1991;84:III236-44.

23. Hashimoto K, Ilstrup DM, Schaff HV. Influence of clinical and hemodynamic variables on risk of supraventricular tachycardia after coronary artery bypass. J Thorac Cardiovasc Surg. 1991;101:56-65.

24. Caretta Q, Mercanti CA, De Nardo D, Chiarotti F, Scibilia G, Reale A, et al. Ventricular conduction defects and atrial fibrillation after coronary artery bypass grafting. Multivariate analysis of preoperative, intraoperative and postoperative variables. Eur Heart J. 1991;12:1107-11.

25. Fuller JA, Adams GG, Buxton B. Atrial fibrillation after coronary artery bypass grafting. Is it a disorder of the elderly? J Thorac Cardiovasc Surg. 1989; 97:821-5.

26. Klemperer JD, Klein IL, Ojamaa K, Helm RE, Gomez M, Isom OW, et al. Triiodothyronine therapy lowers the incidence of atrial fibrillation after cardiac operations. Ann Thorac Surg. 1996;61:1323-7; discussion 1328-9.

27. Villareal RP, Hariharan R, Liu BC, Kar B, Lee VV, Elayda M, Lopez JA, Rasekh A, Wilson JM, Massumi A. Postoperative atrial fibrillation and mortality after coronary artery bypass surgery.J Am Coll Cardiol. 2004 Mar 3;43(5):742-8.

28. Haıssaguerre M, Jaı¨s P, Shah DC, Takahashi A, Hocini M, Quiniou G, et al. Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. N Engl J Med. 1998;339:659-66.

29. Frost L, Christiansen EH, Mølgaard H, Jacobsen CJ, Allermand H, Thomsen PE. Premature atrial beat eliciting atrial fibrillation after coronary artery bypass grafting. J Electrocardiol. 1995;28:297-305.

30. Sato S, Yamauchi S, Schuessler RB, Boineau JP, Matsunaga Y, Cox JL. The effect of augmented atrial hypothermia on atrial refractory period, conduction, and atrial flutter/fibrillation in the canine heart. J Thorac Cardiovasc Surg. 1992; 104:297-306.

31. Cox JL. A perspective of postoperative atrial fibrillation in cardiac operations [Editorial]. Ann Thorac Surg. 1993;56:405-9.

32. Zaman AG, Archbold RA, Helft G, Paul EA, Curzen NP, Mills PG. Atrial fibrillation after coronary artery bypass surgery: a model for preoperative risk stratification. Circulation. 2000;101:1403-8.

33. Kalman JM, Munawar M, Howes LG, Louis WJ, Buxton BF, Gutteridge G, et al. Atrial fibrillation after coronary artery bypass grafting is associated with sympathetic activation. Ann Thorac Surg. 1995;60:1709-15.

34. Smith PK, Buhrman WC, Levett JM, Ferguson TB Jr, Holman WL, Cox JL. Supraventricular conduction abnormalities following cardiac operations. A complication of inadequate atrial preservation. J Thorac Cardiovasc Surg. 1983; 85:105-15.

35. Tchervenkov CI, Wynands JE, Symes JF, Malcolm ID, Dobell AR, Morin JE. Electrical behavior of the heart following high-potassium cardioplegia. Ann Thorac Surg. 1983;36:314-9.

36. Chen XZ, Newman M, Rosenfeldt FL. Internal cardiac cooling improves atrial preservation: electrophysiological and biochemical assessment. Ann Thorac Surg. 1988;46:406-11.

37. Athanasiou T, Aziz O, Mangoush O, Al-Ruzzeh S, Nair S, Malinovski V, Casula R, Glenville B. Does off-pump coronary artery bypass reduce the incidence of post-operative atrial fibrillation? A question revisited Eur J Cardiothorac Surg. 2004 Oct;26(4):701-10.

38. Auer J, Weber T, Berent R, Puschmann R, Hartl P, Ng CK, Schwarz C, Lehner E, Strasser U, Lassnig E, Lamm G, Eber B; Study of Prevention of Postoperative Atrial Fibrillation. A comparison between oral antiarrhythmic drugs in the prevention of atrial fibrillation after cardiac surgery: the pilot study of prevention of postoperative atrial fibrillation (SPPAF), a randomized, placebo-controlled trial. Am Heart J. 2004 Apr;147(4):636-43.

39. Calo L, Bianconi L, Colivicchi F, Lamberti F, Loricchio ML, de Ruvo E, Meo A, Pandozi C, Staibano M, Santini M. N-3 fatty acids for the prevention of atrial fibrillation after coronary artery bypass surgery: a randomized, controlled trial. J Am Coll Cardiol 2005;45:1723–1728.

40. Marchioli R, Barzi F, Bomba E, et al, for the GISSI-Prevenzione Investigators. 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 2002; **105:** 1897–903.

41. Nodari S, Metra M, Milesi G, et al. The role of n-3 PUFAs in preventing the arrhythmic risk in patients with idiopathic dilated cardiomyopathy. Cardiovasc Drugs Ther 2009; **23:** 5–15.

42. GISSI-HF Investigators. Eff ect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial. Lancet 2008; **372:** 1223–30.

43. Leaf A, Albert CM, Josephson M, et al, for the Fatty Acid Anti-arrhythmia Trial Investigators. Prevention of fatal arrhythmias in high-risk subjects by fish oil n-3 fatty acid intake. Circulation 2005; **112:** 2762–68.

44. Brouwer IA, Zock PL, Camm AJ, et al, for the SOFA Study Group. Eff ect of fish oil on ventricular tachyarrhythmia and death in patients with implantable cardioverter defi brillators: the Study on Omega-3 Fatty Acids and Ventricular Arrhythmia (SOFA) randomized trial. JAMA 2006; **295:** 2613–19.

45. Raitt MH, Connor WE, Morris C, et al. Fish oil supplementation and risk of ventricular tachycardia and ventricular fibrillation in patients with implantable defibrillators: a randomized controlled trial. JAMA 2005; **293:** 2884–91.

46. Brouwer IA, Raitt MH, Dullemeijer C, et al. Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defi brillators. Eur Heart J 2009; **30:** 820–26.

47. Leon H, Shibata MC, Sivakumaran S, Dorgan M, Chatterley T, Tsuyuki RT. Eff ect of fi sh oil on arrhythmias and mortality: systematic review. BMJ 2008; 337: 2931.

48. Burr ML, Ashfi eld-Watt PA, Dunstan FD, et al. Lack of benefit of dietary advice to men with angina: results of a controlled trial. Eur J Clin Nutr 2003; **57**:193-200.

49. Coronel R, Wilms-Schopman FJ, Den Ruijter HM, et al. Dietary n-3 fatty acids promote arrhythmias during acute regional myocardial ischemia in isolated pig hearts. Cardiovasc Res 2007; **73:** 386–94.

50. Dhein S, Michaelis B, Mohr FW. Antiarrhythmic and electrophysiological eff ects of long-chain omega-3 polyunsaturated fatty acids. Naunyn-Schmiedeberg's Arch Pharmacol 2005; **371:** 202–11.

51. Den Ruijter HM, Berecki G, Opthof T, Verkerk AO, Zock PL, Coronel R. Pro- and antiarrhythmic properties of a diet rich in fish oil. Cardiovasc Res 2007; **73:** 316–25.

52. London B, Albert C, Anderson ME, et al. Omega-3 fatty acids and cardiac arrhythmias: prior studies and recommendations for future research: 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. Circulation 2007; **116:** e320–35.

53. Xiao YF, Kang JX, Morgan JP, Leaf A. Blocking effects of polyunsaturated fatty acids on Na+ channels in neonatal rat ventricular myocytes. Proc Natl Acad Sci 1995; **92:** 11000–04.

54. Xiao YF, Wright SN, Wang GK, Morgan JP, Leaf A. Fatty acids suppress voltage-gated Na+ currents in HEK293t cells transfected with the alpha subunit of the human cardiac sodium channel. Proc Natl Acad Sci 1998; **95:** 2680–85.

55. Leifert WR, McMurchie EJ, Saint DA. Inhibition of cardiac sodium currents in adult rat myocytes by n-3 polyunsaturated fatty acids. J Physiol 1999; **520:** 671–79.

56. Leifert WR, Jahangiri A, McMurchie EJ. Membrane fluidity changes are associated with the antiarrhythmic eff ects of docosahexaenoic acid in adult rat cardiomyocytes. J Nutr Biochem 2000; **11:** 38–44.

57. Mozaff arian D, Stein PK, Prineas RJ, Siscovick DS. Dietary fish and omega-3 fatty acid consumption and heart rate variability in US adults. Circulation 2008; **117:** 1130–37.

58. Christensen JH, Gustenhoff P, Korup E, et al. Eff ect of fish oil on heart rate variability in survivors of myocardial infarction: a double blind randomized controlled trial. BMJ 1996; **31:** 677–78.

59. O'Keefe JH Jr, Abuissa H, Sastre A, Steinhaus DM, Harris WS. 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. Am J Cardiol 2006; **97:** 1127–30.

60. Mozaff arian D, Geelen A, Brouwer IA, Geleijnse JM, Zock PL, Katan MB. Eff ect of fi sh oil on heart rate in humans: a meta-analysis of randomized controlled trials. Circulation 2005; **112:** 1945–52.

61. Verkerk AO, den Ruijter HM, Bourier J, et al. Dietary fish oil reduces pacemaker current and heart rate in rabbit. Heart Rhythm 2009; **6:** 1485–92.

62. Abdukeyum GG, Owen AJ, McLennan PL. Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning. J Nutr 2008; **138:** 1902–09.

63. Senges S, for the OMEGA Study group. Randomised trial of omega-3 fatty acids on top of modern therapy after acute myocardial infarction: the OMEGA trial. Oral presentation at the Annual Scientifi c Sessions of the American College of Cardiology; Orlando, FL, March 2009

64. Mozaff arian D, Bryson CL, Lemaitre RN, Burke GL, Siscovick DS. Fish intake and risk of incident heart failure. J Am Coll Cardiol 2005; **45:** 2015–21

65. Yamagishi K, Iso H, Date C, et al, for the Japan Collaborative Cohort Study for Evaluation of Cancer Risk Study Group. Fish, omega-3 polyunsaturated fatty acids, and mortality from cardiovascular diseases in a nationwide community-based cohort of Japanese men and women the JACC (Japan Collaborative Cohort Study for Evaluation of Cancer Risk) Study. J Am Coll Cardiol 2008; 52: 988–96.

66. Yamagishi K, Nettleton JA, Folsom AR; ARIC Study Investigators. Plasma fatty acid composition and incident heart failure in middle aged adults: the Atherosclerosis Risk in Communities (ARIC) Study. Am Heart J 2008; **156:** 965–74.

67. Macchia A, Levantesi G, Franzosi MG, et al; GISSI-Prevenzione Investigators. Left ventricular systolic dysfunction, total mortality, and sudden death in patients with myocardial infarction treated with n-3 polyunsaturated fatty acids. Eur J Heart Fail 2005; **7:** 904–09.

68. Brouwer IA, Heeringa J, Geleijnse JM, Zock PL, Witteman JC. Intake of very long-chain n-3 fatty acids from fi sh and incidence of atrial fibrillation. The Rotterdam Study. Am Heart J 2006; **151:** 857–62.

69. Frost L, Vestergaard P. n-3 Fatty acids consumed from fish and risk of atrial fibrillation or flutter: the Danish Diet, Cancer, and Health Study. Am J Clin Nutr 2005; **81:** 50–54.

70. Mozaff arian D, Psaty BM, Rimm EB, et al. Fish intake and risk of incident atrial fibrillation. Circulation 2004; **110:** 368–73.

71. Virtanen JK, Mursu J, Voutilainen S, Tuomainen TP. Serum long chain n-3 polyunsaturated fatty acids and risk of hospital diagnosis of atrial fibrillation in men. Circulation 2009; **120:** 2315–21.

72. Leaf A. Plasma non-esterified fatty acid concentration as a risk factor for sudden cardiac death: the Paris Prospective Study. *Circulation* 2001;104:744 –745.

73. Billman GE, Kang JX, Leaf A. Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. *Circulation* 1999;99:2452–2457.

74. Alberte C, Zipes DP. Use of non-anti-arrhythmic drugs for prevention of sudden cardiac death. *J Cardiovasc Electrophysiol* 2003;14(suppl): S87–S95.

75. Kang JX, Leaf A. Prevention and termination of beta-adrenergic agonist- induced arrhythmias by free polyunsaturated fatty acids in neonatal rat cardiac myocytes. *Biochem Biophys Res Commun* 1995;208: 629–636.

76. Albert CM, Campos H, Stampfer MJ, Ridker PM, Manson JE, Willett WC, et al. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. N Engl J Med 2002;346:1113–8.

77. Kitajka K, Sinclair AJ, Weisinger RS, Weisinger HS, Mathai M, Jayasooriya AP, et al. Effects of dietary omega-3 polyunsaturated fatty acids on brain gene expression. Proc Natl Acad Sci 2004;101: 10931–6.

78. Higdon JV, Liu J, Du SH, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater in vivo lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F(2)-isoprostanes. Am J Clin Nutr 2000;72:714–22.

79. Hansen RA, Ogilvie GK, Davenport DJ, Gross KL, Walton JA, Richardson KL, et al. Duration of effects of dietary fish oil supplementation on serum eicosapentaenoic acid and docosahexaenoic acid concentrations in dogs. Am J Vet Res 1998;59:864–8.

80. Virtanen JK, Mursu J, Voutilainen S, Tuomainen TP. Serum long-chain n-3 polyunsaturated fatty acids and risk of hospital diagnosis of atrial fibrillation in men. Circulation. 2009 Dec 8;120(23):2315-21.

81. Berecki G, Den Ruijter HM, Verkerk AO, Schumacher CA, Baartscheer A, Bakker D, Boukens BJ, van Ginneken AC, Fiolet JW, Opthof T, Coronel R. Dietary fish

oil reduces the incidence of triggered arrhythmias in pig ventricular myocytes. Heart Rhythm. 2007 Nov;4(11):1452-60.

82. Den Ruijter HM, Berecki G, Verkerk AO, Bakker D, Baartscheer A, Schumacher CA, Belterman CN, de Jonge N, Fiolet JW, Brouwer IA, Coronel R. Acute administration of fish oil inhibits triggered activity in isolated myocytes from rabbits and patients with heart failure. Circulation. 2008 Jan 29;117(4):536-44.

83. Hallaq, H., Sellmayer, A., Smith, T. W. & Leaf, A. Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes. (1990) Proc. Natl. Acad. Sci. USA 87, 7834-7838.

84. Hallaq, H., Smith, T. W. & Leaf, A. Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids (1992) Proc. Natl. Acad. Sci.USA 89, 1760-1764.

85. Kang, J. X. & Leaf, A. Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes (1994) Proc. Natl. Acad. Sci. USA 91,9886-9890.

86. Jing X. Kang,Yong-fu Xiao, Alexander Leaf. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. Proc. Natl. Acad. Sci. USA Vol. 92, pp. 3997-4001, April 1995

87. Jennifer C. Macleod, Anthony D.C. Macknight, Glenn C. Rodrigo. The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to ω3 polyunsaturated fatty acids. European Journal of Pharmacology 356-1998.261–270

88. Xiao YF, Kang JX, Morgan JP, Leaf A. Blocking effects of polyunsaturated fatty acids on Na+ channels in neonatal rat ventricular myocytes. Proc Natl Acad Sci 1995;92:11000–4.

89. Xiao YF, Wright SN, Wang GK, Morgan JP, Leaf A. Fatty acids suppress voltage-gated Na+ currents in HEK293t cells transfected with the alpha subunit of the human cardiac sodium channel. Proc Natl Acad Sci 1998;95:2680–5.

90. Jing X. Kang.,Yong-fu Xiao,Alexander Leaf. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. Proc. Natl. Acad. Sci. USA Vol. 92, pp. 3997-4001, April 1995

91. Jennifer C. Macleod, Anthony D.C. Macknight, Glenn C. Rodrigo. The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to ω3 polyunsaturated fatty acids. European Journal of Pharmacology 356_1998.261–270

92. Xiao YF, Ke Q, Wang SY, Auktor K, Yang Y, Wang GK, et al. Single point mutations affect fatty acid block of human myocardial sodium channel alpha subunit Na+ channels. Proc Natl Acad Sci 2001; 98:3606–11.

93. Leifert WR, McMurchie EJ, Saint DA. Inhibition of cardiac sodium currents in adult rat myocytes by n-3 polyunsaturated fatty acids. J Physiol 1999;520:671–9.

94. Verkerk AO, van Ginneken ACG, Berecki G, Den Ruijter HM, Schumacher CA, Veldkamp MW, et al. Incorporated sarcolemmal fish oil fatty acids shorten pig ventricular action potentials. Cardiovasc Res 2006;70:509–20.

95. Leifert WR, Jahangiri A, Saint DA, McMurchie EJ. Effects of dietary n-3 fatty acids on contractility, Na+ and K+ currents in a rat cardiomyocyte model of arrhythmia. J Nutr Biochem 2000;11:382–92.

96. Kang JX. Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. Proc Natl Acad Sci 1995;92:3997–4001.

97. Dhein S, Michaelis B, Mohr FW. Antiarrhythmic and electrophysiological effects of long-chain omega-3 polyunsaturated fatty acids. Naunyn-Schmiedeberg's Arch Pharmacol 2005;371:202–11.

98. Singleton CB, Valenzuela SM, Walker BD, Tie H, Wyse KR, Bursill JA, et al. Blockade by n-3 polyunsaturated fatty acid of the Kv4.3 current stably expressed in Chinese hamster ovary cells. Br J Pharmacol 1999;127:941–8.

99. Macleod JC, Macknight ADC, Rodrigo GC. The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to omega-3 polyunsaturated fatty acids. Eur J Pharmacol 1998; 356:261–70.

100. Xiao YF, Morgan JP, Leaf A. Effects of polyunsaturated fatty acids on cardiac voltage-activated K+ currents in adult ferret cardiomyocytes. Sheng Li Xue Bao 2002;54:271–81.

101. Judé S, Bedut S, Roger S, Pinault M, Champeroux P, White E, et al. Peroxidation of docosahexaenoic acid is responsible for its effects on ITO and ISS in rat ventricular myocytes. Br J Pharmacol 2003;139: 816–22.

102. Antzelevitch C, Sicouri S, Litovsky SH, Lukas A, Krishnan SC, Di Diego JM, et al. Heterogeneity within the ventricular wall. Electrophysiology and pharmacology of epicardial, endocardial, and M cells. Circ Res 1991;69:1427–49.

103. Xiao YF, Morgan JP, Leaf A. Effects of polyunsaturated fatty acids on cardiac voltage-activated K+ currents in adult ferret cardiomyocytes. Sheng Li Xue Bao 2002;54:271–81.

104. Judé S, Bedut S, Roger S, Pinault M, Champeroux P, White E, et al. Peroxidation of docosahexaenoic acid is responsible for its effects on ITO and ISS in rat ventricular myocytes. Br J Pharmacol 2003;139: 816–22.

105. Doolan GK, Panchal RG, Fonnes EL, Clarke AL, Williams DA, Petrou S. Fatty acid augmentation of the cardiac slowly activating delayed rectifier current (IKs) is conferred by hminK. FASEB J 2002;12:1662–4.

106. Hallaq, H., Smith, T. W. & Leaf, A. Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids (1992) Proc. Natl. Acad. Sci.USA 89, 1760-1764.

107. Macleod JC, Macknight ADC, Rodrigo GC. The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to omega-3 polyunsaturated fatty acids. Eur J Pharmacol 1998; 356:261–70.

108. Xiao YF, Gomez AM, Morgan JP, Lederer WJ, Leaf A. Suppression of voltage-gated L-type calcium currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. Proc Natl Acad Sci 1997;94:4182–7.

109. Ferrier GR, Redondo I, Zhu J, Murphy MG. Differential effects of docosahexaenoic acid on contractions and L-type calcium current in adult cardiac myocytes. Cardiovasc Res 2002;54:601–10.

110. Xiao YF, Ke Q, Chen Y, Morgan JP, Leaf A. Inhibitory effect of n-3 fish oil fatty acids on cardiac Na+/Ca2+ exchange currents in HEK293t cells. Biochem Biophys Res Commun 2004;321:116–23.

111. Leifert WR, Dorian CL, Jahangiri A, McMurchie EJ. Dietary fish oil prevents asynchronous contractility and alters Ca2+ handling in adult rat cardiomyocytes. J Nutr Biochem 2001;12:365–76.

112. O'Neill SC, Perez MR, Hammond KE, Sheader EA, Negretti N. Direct and indirect modulation of rat cardiac sarcoplasmic reticulum function by n-3 polyunsaturated fatty acids. J Physiol 2002;538:179–84.

113. Pogwizd SM, Bers DM. Cellular basis of triggered arrhythmias in heart failure. Trends Cardiovasc Med 2004;14:61–6.

114. Xiao YF, Gomez AM, Morgan JP, Lederer WJ, Leaf A. Suppression of voltage-gated L-type Ca2+ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. Proc Natl Acad Sci 1997;94:4182–7.

115. Negretti N, Perez MR, Walker D, O'Neill SC. Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle. J Physiol 2000;523:367–75.

116. Swan JS, Dibb K, Negretti N, O'Neill SC, Sitsapesan R. Effects of eicosapentaenoic acid on cardiac SR Ca2+-release and ryanodine receptor function. Cardiovasc Res 2003;60:337–46.

117. O'Neill SC, Perez MR, Hammond KE, Sheader EA, Negretti N. Direct and indirect modulation of rat cardiac sarcoplasmic reticulum function by n-3 polyunsaturated fatty acids. J Physiol 2002;538:179–84.

118. Honen BN. Suppression of calcium sparks in rat ventricular myocytes and direct inhibition of sheep cardiac RyR channels by EPA, DHA and oleic acid. J Membr Biol 2003;196:95–103.

119. Verkerk AO, Veldkamp MW, Baartscheer A, Schumacher CA, Klopping C, van Ginneken ACG, et al. Ionic mechanism of delayed after-depolarizations in ventricular cells isolated from human end-stage failing hearts. Circulation 2001;104:2728–33.

120. Baartscheer A, Schumacher CA, Belterman CNW, Coronel R, Fiolet JWT. SR calcium handling and calcium after-transients in a rabbit model of heart failure. Cardiovasc Res 2003;58:99–108.

121. Den Ruijter HM, Berecki G, Belterman CNW, Schumacher CA, Baartscheer A, Fiolet JWT, et al. Fish oil reduces the number of calcium after-transients and delayed afterdepolarizations in isolated myocytes from rabbits with heart failure. (Abstract). Circulation 2005;112:1717 [Suppl S].

122. Leifert WR, Dorian CL, Jahangiri A, McMurchie EJ. Dietary fish oil prevents asynchronous contractility and alters Ca2+ handling in adult rat cardiomyocytes. J Nutr Biochem 2001;12:365–76.

123. Guizy M, David M, Arias C, Zhang L, Cofán M, Ruiz-Gutiérrez V, Ros E, Lillo MP, Martens JR, Valenzuela C. Modulation of the atrial specific Kv1.5 channel by the n-3 polyunsaturated fatty acid, alpha-linolenic acid. J Mol Cell Cardiol. 2008 Feb;44(2):323-35.

124. Sarrazin JF, Comeau G, Daleau P, Kingma J, Plante I, Fournier D, Molin F. Reduced incidence of vagally induced atrial fibrillation and expression levels of connexins by n-3 polyunsaturated fatty acids in dogs. J Am Coll Cardiol. 2007 Oct 9;50(15):1505-12.

125. Aviles RJ, Martin DO, Apperson-Hansen C, Houghtaling PL, Rautaharju P, Kronmal RA, Tracy RP, Van Wagoner DR, Psaty BM, Lauer MS, Chung MK. Inflammation as a risk factor for atrial fibrillation. Circulation 2003; 108:3006-10.

126. Calder PC. N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006; 83:1505S-19S.

127. Malek M et al. Heart Rate Variability, Standards of Measurement, Physiological Interpretation, and Clinical Use Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology. Circulation. 1996;93:1043-1065

128. Price CP, Trull AK, Berry D, Gorman EG. Development and validation of a particleenhanced turbidimetric immunoassay for C-reactive protein. J Immunol Methods. 1987; 99:205-11

129. Eda S, Kaufmann J, Roos W, Pohl S. Development of a new microparticle-enhanced turbidimetric assay for C-reactive protein with superior features in analytical sensitivity and dynamic range. J Clin Lab Anal. 1998; 12:137-44.

130. Bustin SA, Benes V, Nolan T and Pfaffl MW. Quantitative real-time RT-PCR – a perspective Journal of Molecular Endocrinology (2005) 34 597-601

131. Chandler NJ, Greener ID, Tellez JO, Inada S, Musa H, Molenaar P, Difrancesco D, Baruscotti M, Longhi R, Anderson RH, Billeter R, Sharma V, Sigg DC, Boyett MR, Dobrzynski H. Molecular architecture of the human sinus node: insights into the function of the cardiac pacemaker. Circulation. 2009 Mar 31;119(12):1562-75.

132. Dobrev D, Friedrich A, Voigt N, Jost N, Wettwer E, Christ T, Knaut M, Ravens U. The G protein-gated potassium current I(K,ACh) is constitutively active in patients with chronic atrial fibrillation. Circulation. 2005 Dec 13;112(24):3697-706.

133. Hale CC, Slaughter RS, Ahrens DC, Reeves JP.Identification and partial purification of the cardiac sodium-calcium exchange protein. Proc Natl Acad Sci U S A. 1984 Nov;81(21):6569-73.

134. Kirchberger MA, Antonetz T. Phospholamban: dissociation of the 22,000 molecular weight protein of cardiac sarcoplasmic reticulum into 11,000 and 5,500 molecular weight forms.Biochem Biophys Res Commun. 1982 Mar 5;105(1):152-6

135. Lokuta AJ, Maertz NA, Meethal SV, Potter KT, Kamp TJ, Valdivia HH, Haworth RA. Increased nitration of sarcoplasmic reticulum Ca2+-ATPase in human heart failure. Circulation. 2005 Mar 1;111(8):988-95.

136. Takasago T, Imagawa T, Shigekawa M. Phosphorylation of the cardiac ryanodine receptor by cAMP-dependent protein kinase. J Biochem. 1989 Nov;106(5):872-7.

137. Heidt MC, Vician M, Stracke SK, Stadlbauer T, Grebe MT, Boening A, Vogt PR, Erdogan A. Beneficial effects of intravenously administered N-3 fatty acids for the prevention of atrial fibrillation after coronary artery bypass surgery: a prospective randomized study. Thorac Cardiovasc Surg. 2009 Aug;57(5):276-80.

138. Heidarsdottir R, Arnar DO, Skuladottir GV, Torfason B, Edvardsson V, Gottskalksson G, Palsson R, Indridason OS. Does treatment with n-3 polyunsaturated fatty acids prevent atrial fibrillation after open heart surgery? Europace. 2010 Mar;12(3):356-63.

139. Sandesara C, Chung MK, Van Wagoner DR, et al. Fish oil to inhibit supraventricular arrhythmias after cardiac surgery: the FISH Trial. In: Heart Rhythm Society's 31st Annual Scientific Sessions. 12e15 May 2010, Denver, Late-Breaking Clinical Trials II.

140. Liu T, Korantzopoulos P, Shehata M, Li G, Wang X, Kaul S. Prevention of atrial fibrillation with omega-3 fatty acids: a meta-analysis of randomised clinical trials. Heart. 2011 Jul;97(13):1034-40.

141. Saravanan P, Bridgewater B, West AL, O'Neill SC, Calder PC, Davidson NC. Omega-3 fatty acid supplementation does not reduce risk of atrial fibrillation after coronary artery bypass surgery: a randomized, double-blind, placebo-controlled clinical trial. Circ Arrhythm Electrophysiol. 2010 Feb 1;3(1):46-53.

142. Erdogan A, Bayer M, Kollath D, et al. OMEGA AF study: polyunsatuated fatty acids (PUFA) for prevention of atrial fibrillation relapse after successful external cardioversion. Heart Rhythm 2007;4:S185e6.

143. Margos P, Leftheriotis D, Katsouras G, et al. Influence of n-3 fatty acids intake on secondary prevention after cardioversion of persistent atrial fibrillation to sinus rhythm. Europace 2007;9:iii51.

144. Nodari S, Triggiani M, Foresti A, et al. Use of N-3 polyunsaturated fatty acids to maintain sinus rhythm after conversion from persistent atrial fibrillation. A prospective randomized study. J Am Coll Cardiol 2010;55:A2.E14.

145. Bianconi L, Calo` L, Mennuni M, et al. n-3 Poly-unsaturated fatty acids for the prevention of arrhythmia recurrence after electrical cardioversion of chronic persistent atrial fibrillation: a randomized, double-blind, multicenter study. Europace 2011;13:174e81

146. Kowey PR, Reiffel JA, Ellenbogen KA, et al. Efficacy and safety of prescription omega-3 fatty acids for the prevention of recurrent symptomatic atrial fibrillation: a randomized controlled trial. JAMA 2010;304:2363e72.

147. Mozaffarian D, Psaty BM, Rimm EB, Lemaitre RN, Burke GL, Lyles MF, Lefkowitz D, Siscovick DS. Fish intake and risk of incident atrial fibrillation. Circulation. 2004 Jul 27;110(4):368-73.

148. Lau DH, Psaltis PJ, Carbone A, Kelly DJ, Mackenzie L, Worthington M, Metcalf RG, Kuklik P, Nelson AJ, Zhang Y, Wong CX, Brooks AG, Saint DA, James MJ, Edwards J, Young GD, Worthley SG, Sanders P. Atrial protective effects of n-3 polyunsaturated fatty acids: a long-term study in ovine chronic heart failure. Heart Rhythm. 2011 Apr;8(4):575-82.

149. Virtanen JK, Mursu J, Voutilainen S and Tuomainen TP, Serum long-chain n-3 polyunsaturated fatty acids and risk of hospital diagnosis of atrial fibrillation in men, Circulation 120 (2009), pp. 2315–2321

150. Anselmi A, Possati G, Gaudino M. Postoperative inflammatory reaction and atrial fibrillation: simple correlation or causation? Ann Thorac Surg 2009;88:326–33.

151. Liu T, Li L, Korantzopoulos P, et al. Statin use and development of atrial fibrillation: a systematic review and meta-analysis of randomized clinical trials and observational studies. Int J Cardiol 2008;126:160–70.

152. Mori TA, Beilin LJ. Omega-3 fatty acids and inflammation. Curr Atheroscler Rep 2004;6:461–7.

153. Mas E, Woodman RJ, Burke V, et al. The omega-3 fatty acids EPA and DHA decrease plasma F(2)-isoprostanes: results from two placebo-controlled interventions. Free Radic Res 2010;44:983–90.

154. Sheng X, Scherlag BJ, Yu L, Li S, Ali R, Zhang Y, Fu G, Nakagawa H, Jackman WM, Lazzara R, Po SS. Prevention and reversal of atrial fibrillation inducibility and autonomic remodeling by low-level vagosympathetic nerve stimulation. J Am Coll Cardiol. 2011 Feb 1;57(5):563-71.

155. Lim PB, Malcolme-Lawes LC, Stuber T, Wright I, Francis DP, Davies DW, Peters NS, Kanagaratnam P. Intrinsic cardiac autonomic stimulation induces pulmonary

vein ectopy and triggers atrial fibrillation in humans. J Cardiovasc Electrophysiol. 2011 Jun;22(6):638-46

156. Xia C, Xia J, Shui Z, Liu J, Sun W, Du Y, Hao G. Effects of acetylcholine on electrical remodeling of human atrial fibers. J Huazhong Univ Sci Technolog Med Sci. 2011 Apr;31(2):164-8.

157. Bauernschmitt R, Malberg H, Wessel N, Kopp B, Schirmbeck EU, Lange R. Impairment of cardiovascular autonomic control in patients early after cardiac surgery. Eur J Cardiothorac Surg. 2004 Mar;25(3):320-6.

158. Christensen JH, Svensson M, Strandhave C, Madsen T, Schmidt EB. N-3 fatty acids and cardiac autonomic function in humans. Cell Mol Biol. 2010 Feb 25;56(1):131-9.

159. Christensen JH, Schmidt EB. Autonomic nervous system, heart rate variability and n-3 fatty acids. J Cardiovasc Med (Hagerstown). 2007 Sep;8 Suppl 1:S19-22.

160. Sagara M, Njelekela M, Teramoto T, Taguchi T, Mori M, Armitage L, Birt N, Birt C, Yamori Y. Effects of docosahexaenoic Acid supplementation on blood pressure, heart rate, and serum lipids in Scottish men with hypertension and hyper-cholesterolemia. Int J Hypertens. 2011 Mar 8;2011:809198.

161. Christensen JH, Svensson M, Strandhave C, Madsen T, Schmidt EB. N-3 fatty acids and cardiac autonomic function in humans. Cell Mol Biol. 2010 Feb 25;56(1):131-9.

162. Mozaffarian D, Prineas RJ, Stein PK, Siscovick DS. Dietary fish and n-3 fatty acid intake and cardiac electrocardiographic parameters in humans. J Am Coll Cardiol. 2006 Aug 1;48(3):478-84.

163. Mozaffarian D, Stein PK, Prineas RJ, Siscovick DS. Dietary fish and omega-3 fatty acid consumption and heart rate variability in US adults. Circulation. 2008 Mar 4;117(9):1130-7.

164. P.E. Dilaveris, E.J. Gialafos, S.K. Sideris, A.M. Theopistou, G.K. Andrikopoulos, M. Kyriakidis, J.E. Gialafos and P.K. Toutouzas, Simple electrocardiographic markers for the prediction of paroxysmal idiopathic atrial fibrillation, Am Heart J 135 (1998), pp. 733–738.

165. Magnani JW, Johnson VM, Sullivan LM, Gorodeski EZ, Schnabel RB, Lubitz SA, Levy D, Ellinor PT, Benjamin EJ. P wave duration and risk of longitudinal atrial fibrillation risk in persons ≥60 years old (from the Framingham Heart Study). Am J Cardiol. 2011 Mar 15;107(6):917-921.

166. A.E. Buxton and M.E. Josephson, The role of P wave duration as a predictor of postoperative atrial arrhythmias, Chest 80 (1981), pp. 68–73.

167. P. Dilaveris, V. Batchvarov, J. Gialafos and M. Malik, Comparison of different methods for manual P wave duration measurement in 12-lead electrocardiograms, Pacing Clin Electrophysiol 22 (1999), pp. 1532–1538.

168. P.E. Dilaveris, P. Färbom, V. Batchvarov, A. Ghuran and M. Malik, Circadian behavior of P-wave duration, P-wave area, and PR interval in healthy subjects, Ann Noninvas Electrocardiol 6 (2001), pp. 92–97.

169. Brouwer IA, Raitt MH, Dullemeijer C, Kraemer DF, Zock PL, Morris C, Katan MB, Connor WE, Camm JA, Schouten EG, McAnulty J. Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators. Eur Heart J. 2009 Apr;30(7):820-6.

170. León H, Shibata MC, Sivakumaran S, Dorgan M, Chatterley T, Tsuyuki RT. Effect of fish oil on arrhythmias and mortality: systematic review. BMJ. 2008 Dec 23;337:a2931.

171. Saravanan P, Davidson NC, Schmidt EB, Calder PC. Cardiovascular effects of marine omega-3 fatty acids. Lancet. 2010 Aug 14; 76(9740):540-50.

172. Metcalf RG, James MJ, Gibson RA. Effects of fish-oil supplementation on myocardial fatty acids in humans. Am J Clin Nutr 2007;85:1222–8

173. Harris WS. n-3 fatty acids and serum lipoproteins: animal studies. Am J Clin Nutr. 1997 May; 65(5 Suppl):1611S-1616S.

174. Richardson ES, Iaizzo PA, Xiao YF. Electrophysiological mechanisms of the anti-arrhythmic effects of omega-3 fatty acids. J Cardiovasc Transl Res. 2011 Feb;4(1):42-52.

175. Xiao, Y. F., Ke, Q., Wang, S. Y., .Single point mutations affect fatty acid block of human myocardial sodium channel alpha subunit Na+channels. Proceedings of the National Academy of Sciences of the United States of America, (2001) 98, 3606–3611.

176. Xiao, Y. F., Wright, S. N., Wang, G. K., Morgan, J. P., & Leaf, A. Coexpression with beta(1)-subunit modifies the kinetics and fatty acid block of hH1(alpha) Na(+) channels. American Journal of Physiology. Heart and Circulatory Physiology, (2000) 279, H35–H46.

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

178. Xiao, Y. F., Sigg, D. C., & Leaf, A. The antiarrhythmic effect of n-3 polyunsaturated fatty acids: modulation of cardiac ion channels as a potential mechanism. The Journal of Membrane Biology, (2005) 206, 141–154.

179. Lundbaek, J. A., Birn, P., Girshman, J., Hansen, A. J., & Andersen, O. S. (1996). Membrane stiffness and channel function. Biochemistry, 35, 3825–3830.

180. Leaf, A., & Xiao, Y. F. (2001). The modulation of ionic currents in excitable tissues by n-3 polyunsaturated fatty acids. The Journal of Membrane Biology, 184, 263–271.

181. Jansen JA, van Veen TA, de Bakker JM, van Rijen HV. Cardiac connexins and impulse propagation. J Mol Cell Cardiol. 2010 Jan;48(1):76-82.

182. Polontchouk L, Haefliger JA, Ebelt B, Schaefer T, Stuhlmann D, Mehlhorn U, Kuhn-Regnier F, De Vivie ER, Dhein S. Effects of chronic atrial fibrillation on gap junction distribution in human and rat atria. J Am Coll Cardiol. 2001 Sep;38(3):883-91.

183. Sarrazin JF, Comeau G, Daleau P, Kingma J, Plante I, Fournier D, Molin F. Reduced incidence of vagally induced atrial fibrillation and expression levels of connexins by n-3 polyunsaturated fatty acids in dogs. J Am Coll Cardiol. 2007 Oct 9;50(15):1505-12.

184. Greiser M, Lederer WJ, Schotten U. Alterations of atrial Ca(2+) handling as cause and consequence of atrial fibrillation. Cardiovasc Res. 2011 Mar 1;89(4):722-33.

185. Bers DM. Excitation-Contraction Coupling and Cardiac Contractile Force. Dordrecht, the Netherlands: Kluwer Academic Publishers, 2001.

186. Yeh YH, Wakili R, Qi XY, Chartier D, Boknik P, Kääb S, Ravens U, Coutu P, Dobrev D, Nattel S. Calcium-handling abnormalities underlying atrial arrhythmogenesis and contractile dysfunction in dogs with congestive heart failure. Circ Arrhythm Electrophysiol. 2008 Jun 1;1(2):93-102.

187. Zhang Y, Fraser JA, Jeevaratnam K, Hao X, Hothi SS, Grace AA, Lei M, Huang CL. Acute atrial arrhythmogenicity and altered Ca(2+) homeostasis in murine RyR2-P2328S hearts. Cardiovasc Res. 2011 Mar 1;89(4):794-804.

188. Greiser M, Neuberger HR, Harks E, El-Armouche A, Boknik P, de Haan S, Verheyen F, Verheule S, Schmitz W, Ravens U, Nattel S, Allessie MA, Dobrev D, Schotten U. Distinct contractile and molecular differences between two goat models of atrial dysfunction: AV block-induced atrial dilatation and atrial fibrillation. J Mol Cell Cardiol. 2009 Mar;46(3):385-94.

189. Vest JA, Wehrens XH, Reiken SR, Lehnart SE, Dobrev D, Chandra P, Danilo P, Ravens U, Rosen MR, Marks AR. Defective cardiac ryanodine receptor regulation during atrial fibrillation. Circulation. 2005 Apr 26;111(16):2025-32.

190. Dobrev D, Voigt N, Wehrens XH. The ryanodine receptor channel as a molecular motif in atrial fibrillation: pathophysiological and therapeutic implications. Cardiovasc Res. 2011 Mar 1;89(4):734-43.

191. Wit AL, Rosen MR. Afterdepolarizations and triggered activity: distinction from automaticity as an arrhythmogenic mechanism. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan HE, ed. The Heart and Cardiovascular System. 2nd ed. New York, NY: Raven Press; 1992: 2113–2163.

192. Dilaveris PE, Gialafos JE. P-wave dispersion: a novel predictor of paroxysmal atrial fibrillation. Ann Noninvasive Electrocardiol. 2001 Apr;6(2):159-65.

193. Glotzer TV, Daoud EG, Wyse DG, Singer DE, Ezekowitz MD, Hilker C, Miller C, Qi D, Ziegler PD. The relationship between daily atrial tachyarrhythmia burden from implantable device diagnostics and stroke risk: the TRENDS study. Circ Arrhythm Electrophysiol. 2009 Oct;2(5):474-80.

194. Szentandrássy N, Pérez-Bido MR, Alonzo E, Negretti N, O'Neill SC. Protein kinase A is activated by the n-3 polyunsaturated fatty acid eicosapentaenoic acid in rat ventricular muscle. J Physiol. 2007 Jul 1;582 (Pt 1):349-58.

Publications

Saravanan P, Davidson NC, Schmidt EB, Calder PC. Cardiovascular effects of marine omega-3 fatty acids. Lancet. 2010 Aug 14;376(9740):540-50.

Saravanan P, Bridgewater B, West AL, O'Neill SC, Calder PC, Davidson NC. Omega-3 fatty acid supplementation does not reduce risk of atrial fibrillation after coronary artery bypass surgery: a randomized, double-blind, placebo-controlled clinical trial. Circ Arrhythm Electrophysiol. 2010 Feb 1;3(1):46-53.

Scientific Letters

Saravanan P, Davidson NC. Fish oil and arrhythmias. Pro-arrhythmic effects of fish oils. BMJ. 2009 Feb 2;338:b393

Saravanan P, Davidson NC. The role of omega-3 fatty acids in primary prevention of coronary artery disease and in atrial fibrillation is controversial. J Am Coll Cardiol. 2010 Jan 26;55(4):410-1.

Presentations & Posters

Young Investigator of the year- Presentation: Heart Rhythm Congress- Birmingham-UK- 2010: Omega-3 fatty acids alter expression levels of calcium handling proteins in human heart.

Saravanan P, Gately M, Dobrzynski H, Davidson NC. Relationship between expression levels of atrial connexins, P wave dispersion and risk of post-operative atrial fibrillation following CABG. Heart Rhythm Congress- Birmingham, UK- 2010

Saravanan P, Pollock R, Davidson NC, Trafford A, Dobrzynski H. Omega-3 Fatty Acids Upregulate M2 Receptors In Human Atrium & Improve Heart Rate Variability. Abstract- Poster presentation at Heart Rhythm Society- 31st Annual Conference, 2010, Denver, US

Saravanan P, Pollock R, O'neill SC, Davidson N, Dobrzynski H. Fish oil supplementation increases expression of m-RNA of muscarinic receptors in human right atrium and influences heart rate variability. Abstract- Poster presentation at EUROPACE 2009- Berlin, Germany.

Saravanan P, Gately M, O'neill SC, Davidson N Fish oils supplementation has no influence on ECG-P wave- arrhythmic markers in patients undergoing corornary bypass surgery. Abstract- Poster presentation at EUROPACE 2009- Berlin, Germany

Saravanan P, O'Neill SC, Bridgewater B, Davidson NC. Fish oils supplementation does not reduce risk of atrial fibrillation following coronary artery bypass surgery. Abstract- Poster presentation at Heart Rhythm Society- 30th Annual Conference, 2009, Boston, US.

Appendices

Appendix-1

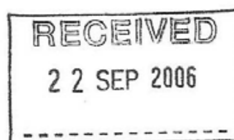
Trent Multi-centre Research Ethics Committee

Chairman: Dr Robert Bing
Co-ordinator: Ms Jill Marshall

Derwent Shared Services
Laurie House
Colyear Street
Derby
DE1 1LJ

Telephone: 01332 868905
Fax: 01332 868930
Email: jill.marshall@derwentsharedservices.nhs.uk

20 September 2006



Dr Neil Davidson
Consultant Cardiologist
Wythenshawe Hospital
Department of Cardiology
Wythenshawe Hospital
Southmoor road, Manchester
M23 9LT

Dear Dr Davidson

Full title of study:	**How does fish oil supplementation protect against atrial fibrillation following coronary artery by-pass surgery? - A cellular study**
REC reference number:	**06/MRE04/58**
Protocol number:	**Designated Version 2 August 2006**
EudraCT number:	**2006-001451-35**

Thank you for your letter of 30 August 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for any other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		30 August 2006
Investigator CV	Mr Stephen O'Neill	
Investigator CV	Dr Neil Davidson	
Protocol	Designated Version 2 August 2006	
Covering Letter		10 July 2006
Letter from Sponsor		11 July 2006
Peer Review	Reviewer's Assessment Form	
Letter of invitation to participant	Designated Version 1	09 July 2006
Participant Information Sheet	3	30 August 2006
Participant Consent Form	Designated Version 2	30 August 2006
Response to Request for Further Information		30 August 2006
summary of the Study Protocol	Designated Version 1	09 July 2006
Letter from Funder	British Heart Foundation	03 May 2006
GP Letter	Designated Version 1	09 July 2006
Request Form to MHRA		

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

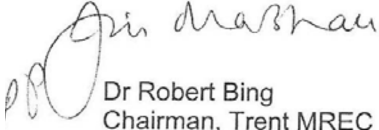
The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/MRE04/58	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely



Dr Robert Bing
Chairman, Trent MREC

Enclosures: *Standard approval conditions [SL-AC]*
Site approval form Issue 1 dated 20 September 2006

Copy to: Dr Andrew Maines
South Manchester University Hospitals NHS Trust
Wythenshawe Hospital
Southmoor Road
Manchester

Clinical Trials Unit, MHRA - via email

Appendix-2

National Research Ethics Service
Trent Research Ethics Committee
Derwent Shared Services
Laurie House
Colyear Street
Derby
DE1 1LJ

Tel: 01332 868842
Fax: 01332 868930

11 February 2008

Dr Neil Davidson
Consultant Cardiologist
Department of Cardiology
Wythenshawe Hospital
Southmoor road, Manchester
M23 9LT

Dear Dr Davidson

Study title: **How does fish oil supplementation protect against atrial fibrillation following coronary artery by-pass surgery? - A cellular study**
REC reference: **06/MRE04/58**
Protocol number: **Designated Version 2 August 2006**
EudraCT number: **2006-001451-35**

Amendment number: **Amendment No.1**
Amendment date: **10 December 2007**

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 07 February 2008.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Covering Letter		30 November 2007
Annex 2 Notification of Amendment (CTIMPs)	Amendment No.1	10 December 2007

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

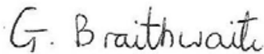
This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/MRE04/58:	**Please quote this number on all correspondence**

Yours sincerely



Mrs Gill Braithwaite
Committee Co-ordinator

E-mail: gill.braithwaite@derwentsharedservices.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

Copy to: Clinical Trials Unit, MHRA (emailed)
Dr Andrew Maines, South Manchester University Hospitals NHS trust

Appendix-3

12-JAN-07 09:48 01612912289 P.02

Safeguarding public health

MHRA

Direct Line: 0207 084-2456
Facsimile: 0207 084-2443
Room 12- 242

Our Reference: 21463/0206/001-0001
Eudract Number: 2006-001451-35

Dr N Davidson
SOUTH MANCHESTER UNIVERSITY HOSPITALS NHS TRUST
DEPARTMENT OF CARDIOLOGY
SOUTHMOOR ROAD
MANCHESTER
M26 9LT
UNITED KINGDOM

please copy for Saravanan (he will collect).

28/12/2006

Dear Dr N Davidson

THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS 2004 S.I. 1031
Product Type: Product with Special Characteristics
Product: OMACOR
Protocol number: 2006CD004

NOTICE OF ACCEPTANCE OF AMENDED REQUEST

I am writing to confirm that the Licensing Authority, acting under regulation 18(6)(b) or (c), or 19(7)(a) or (8), or 20(4)(a) or (5), according to the type of medicinal product involved[1], accepts your amended request to carry out a clinical trial in accordance with your application 19/12/2006 subject to you receiving a favourable opinion from the relevant ethics committee in accordance with regulation 15(1). You may therefore carry out the trial as notified, but I must remind you of the Authority's powers under regulation 31 to suspend or terminate a clinical trial if the conditions set out in regulation 31(1)(a) and (b) are satisfied

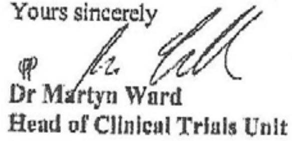
Remarks:
* The method of blinding the active product should be confirmed before the study commences.
* Further information on the composition, method of manufacture and release testing of the placebo product should be provided before the study commences.

[1] The Licensing Authority's authorisation powers for clinical trials are regulation 18 for those involving general medicinal products, regulation 19 for those involving medicinal products for gene therapy etc., and regulation 20 for those involving medicinal products with special characteristics.

Medicines and Healthcare products Regulatory Agency

The authorisation is effective from the date of this letter and may continue under this authorisation. In accordance with regulation 27, you must notify the Licensing Authority within 90 days of the conclusion of the trial, that has ended.

Yours sincerely



Dr Martyn Ward
Head of Clinical Trials Unit

Appendix-4

Version: 4
Date: 12.10.2006
Project ID: 2006CD004

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT
Tel: 0161 998 7070

Part-1

1. Study title

"How does fish oil supplementation protect against atrial fibrillation following coronary artery bypass surgery ?- A cellular study."

2. Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of the study?

This study is designed to investigate the benefits of fish oils in reducing electrical disturbances in the heart called 'Arrythmias'. We are particularly evaluating the benefits in suppressing a common form of heart rhythm disturbance called 'Atrial Fibrillation (AF)'. We will also be studying the changes in the electrical properties of individual heart cells that would confer such protection.

4. Why have I been chosen?

We are aiming to include 300 patients who are undergoing heart by-pass operation in isolation and have never had AF before. You were chosen mainly based on these criteria.

5. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

6. What will happen to me if I take part?

If you decide to take part, we would collect a blood sample (10mls of blood in addition to the sample for pre-operative routine blood tests). We would then allocate you randomly into one of the two groups to receive either 2 capsules of 1g equivalent of fish oil extract per day or an inert placebo (A placebo is a "dummy treatment", which looks like the genuine medicine but contains no active ingredient). Neither you nor the research team will know which group you have been allocated to (double blind). You will need to take these capsules daily until 5 days following your heart

1

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT
Tel: 0161 998 7070

11. What are the other possible disadvantages and risks of taking part?
If you decide to take part, you may have to make an extra visit to the hospital prior to your planned surgery. We would aim to avoid this by doing the research scan as a part of your routine scan that you will have done prior to any heart operation. However, If you had to make any extra visit/s for the purpose of research then we would aim to organise this according to your convenience.

12. What are the possible benefits of taking part?
We believe that patients in the active treatment group receiving fish oil supplements may get some benefit in the form of reduced incidence of heart rhythm disturbances following the surgery. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients who are undergoing this surgery better.

13. What happens when the research study stops?
We will communicate the results of the trial to yourself, your general practitioner in addition to the cardiologist and the cardiac surgeon involved in your care. They will then decide, based on the evidence provided to them, on the need for making any changes to your treatment. You will be able to discuss this with the doctors involved in your care.

14 What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

15. Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

16. Contact Details:
Dr. P. Saravanan, Research Registrar, Department of Cardiology, Wythenshawe Hospital, Southmoor road, Manchester, M26 9LT
Phone: 0161 291 2390. e-mail: *palaniappan.saravanan@smuht.nwest.nhs.uk*

This completes Part 1 of the Information Sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Version: 4
Date: 12.10.2006
Project ID: 2006CD004

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT
Tel: 0161 998 7070

Part 2
Study title
How does fish oil supplementation protect against atrial fibrillation following coronary artery by-pass surgery? - A cellular study.

17. What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interest to withdraw you from the study. He will explain the reasons and arrange for your care to continue.

18. What will happen if I don't want to carry on with the study?
You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

19. What if there is a problem?
Complaints:
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Contact number). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure.
All such complaints should be directed to The Complaints Co-ordinator, Complaints and Legal Services, 3rd Floor Tower Block, Wythenshawe Hospital, Southmoor Road, Manchester. M23 9LT Phone 0161 291 2033

Harm:
In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against South Manchester University Hospitals NHS Trust but you may have to pay your legal costs.. The normal National Health Service complaints mechanisms will still be available to you.

20. Will my taking part in this study be kept confidential?
All information, which is collected about you during the course of the research, will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it.

...Manchester NHS
NHS Foundation Trust

Version: 4
Date: 12.10.2006
Project ID: 2006CD004

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT
Tel: 0161 998 7070

All data will be collected by the research registrar and when applicable by the research nurse. The data thus obtained will be made anonymous by removal of all personal details. It will then be awarded a code by which this data will be identified by everyone involved in the study. The details of the personal identity and the relevant code will be kept safe in a file by the research registrar. This will be stored safely in the cardiology research office at Wythenshawe Hospital, Manchester. At the end of the study, the data will be stored safely and kept in the custody of the R&D division of South Manchester University Hospitals NHS trust for a period of 25 years.

Involvement of the General Practitioner/Family doctor (GP)
We would like your permission to inform your GP about your participation in the trial. We would also wish to inform other medical practitioners (such as your heart surgeon) not involved in the research but are currently involved in your care.

21. What will happen to any samples I give?
We would request you to agree to provide the blood samples and the tissue sample as a gift to the research team. All the samples will be safely disposed off once analysis is completed. No sample will be retained or stored for future use.

22. Will any genetic tests be done?
No, We do not intend to do any genetic studies on this trial.

23. What will happen to the results of the research study?
The study is estimated to conclude in September 2009. We will be able to analyse and publish the results shortly after that. This is likely to be in the form of a research article in scientific journal/s and/or medical conferences. Your identity will not be disclosed in any of these publications. We will inform all the participants about the outcome of this study and also the details about which arm of the study you were in.

24. Who is organising and funding the research?
This study is being organised jointly by the South Manchester University Hospitals NHS trust and The University of Manchester and is being funded by the 'British Heart Foundation'. The British Heart Foundation will pay the salary of the research doctor.

25. Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the Trent Multi-centre Research Ethics Committee.

Thank you for considering to take part in our study. Should you decide to take part, you will be given this information sheet (parts 1&2) and a copy of the signed consent form to keep.

Appendix-5

Project ID: 2006CD004
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: How does fish oil supplementation protect against atrial fibrillation following coronary artery by-pass surgery? - A cellular study.

Name of Researcher: Dr. Neil Davidson

Please initial box

1. I confirm that I have read and understand the information sheet dated .... (version ....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐

________________	________________	________________
Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

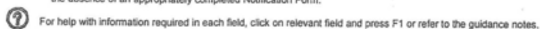
Appendix-6

The Universi of Manchest

Pan-Manchester R&D Notification Form

This **three-page** form must be fully completed and used to inform The University and NHS Trust(s) of all research, whether internally or externally funded. It must be signed by:

- An R&D Office on behalf of the NHS Trust where staff are to be based and resources/facilities will be used[1].
- ALL the Heads of Departments or an Authorised Signatory of each University Department or Division/ School to receive financial credit from such an award. The Faculty of Medical and Human Sciences Research Office will be unable to authorise any proposal in the absence of an appropriately completed Notification Form.



For help with information required in each field, click on relevant field and press F1 or refer to the guidance notes.

1) Project Details			
Full Title of the Project: Please use the same title as used in ethics application form.	HOW DOES FISH OIL SUPPLEMENTATION PROTECT AGAINST ATRIAL FIBRILLATION FOLLOWING CORONARY ARTERY BY-PASS SURGERY – A CELLULAR STUDY		
Sponsor of Project:	The University of Manchester If 'Other' please specify:		
Funder of Project (if different to Sponsor):	British Heart Foundation	Total Project Value (£s)	~~197768.41~~ 182150.35
Name of Organisation receiving/ administering funds:	University of Manchester	Is this a grant application? Has a grant been awarded?	☒ Yes ☐ No ☐ Yes ☒ No
Intended Start Date: (dd/mm/yy)	01/06/06	Intended End Date: (dd/mm/yy)	31/05/09

2) Location	
Sites (Facility and institution(s)) where the research will be conducted:	Stopford Building

3) Ethics (For further information regarding NHS Research Ethics Committees, visit www.corec.org.uk)			
Is a favourable ethics opinion required:	☒ Yes ☐ No	Date Submitted (dd/mm/yy):	01/03/06
If a favourable ethics opinion has already been obtained, please give:			
Committee Name:		Ref No:	
LREC:			
MREC:			
University Ethics Committee:	☐ Yes ☐ No		

4) Type of research	
Is the research a multi-centre project?	☒ Yes ☐ No
If 'Yes' please specify name of Lead Organisation:	**University of Manchester**
Name of Chief Investigator if different to Principal Investigator (given below)	

5) Principal Investigator (PI)		
Name: Title Dr	Forename(s):Stephen	Surname:O'Neill
Tel: 0161 275 7968	Fax: 0161 275 7069	E-mail: mdsssco@manchester.ac.uk
Job Title: Senior Lecturer	Employer Name: The University of Manchester If Other (please specify):	Honorary Contract held? ☐ Yes ☒ No If 'Yes' name issuing organisation:
NHS Trust Department:	University Division/ School Research Group Medicine - Cardiovascular and Endocrine Sciences	Credit share of funding (%):
University staff only: % of effort to be spent on this project: 15%		

[1] Please note that some R&D offices may require additional information before Trust approval can be given.

Page 1 of 3

Appendix-7

Research & Development Directorate
Ground Floor, Education & Research Centre, Wythenshawe Hospital
Tel: 0161 291 5777/5770, Fax: 5771
Email: andrew.maines@manchester.ac.uk

NHS Trust

Ref: ProjApp/*Apprv2006CD004*

25th April 2007

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT

Tel: 0161 998 7070

Dr Neil Colin Davidson
Consultant Cardiologist
Department of Cardilogy
Wythenshawe Hospital

Dear Dr Davidson

Re: How Does Fish Oil Supplementation Protect Against Atrial Fibrillation Following Coronary Artery Bypass

R&D Ref: 2006CD004
Protocol: Version 2
REC Ref: 06/MRE04/58

Thank you for providing us with all the necessary documentation. This research project has now been given R&D Management Approval. Please find enclosed signed copies of the Trial Agreement. Please sign in the approrite places, and return one to R&D and retain one in the Trial Master File.

I have enclosed some labels for you to affix to any Trust hospital casenotes used in your research project.

This is a requirement of the Trust Casenote Destruction Policy. These labels will ensure that casenotes used for research projects, which involve the treatment of patients, are kept for 20 years as recommended by the Medical Research Council.

It is your responsibility to ensure that these labels are affixed on the top left-hand corner of the INSIDE COVER of each casenote. We will provide you with additional labels; please contact us when you require them.

We are required by the Department of Health to report research carried out within this Trust to the National Research Register (NRR). This is carried out on a quarterly basis, ***and is published on the Internet***. The details for this project are shown on the enclosed summary. If you have any changes to make, or **you do not wish these details to appear on the NRR**, please reply to me at the address shown above.

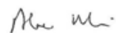
Please note it is a requirement of the approval given by the Trust that the research project is being conducted in line with the guidance given within the Research Governance Framework as issued by the DH: (from the R&D website www.researchdirectorate.org.uk click on the link 'Carrying out research). I have enclosed a summary of investigator responsibilities under the terms of the framework and would be grateful if you could take the time to read this.

May I also draw to your attention the need to comply with the Health & Safety at Work Act, the Data Protection Act and the Human Tissue Act 2004.

I would also be grateful if you could supply us with copies of any substantial amendments submitted to the REC throughout the course of the study, along with a copy of the REC end of study report when it has been completed.

Thank you for your assistance. If you require any further information please do not hesitate to contact us on the above numbers.

Yours sincerely



Dr Andrew Maines
Head of Research & Development
Enc.

Appendix-8 (composition of n-3 PUFA supplemented rat diet)

AIN-93G w/ 40% Kcal From Fish Oil — 5S64

DESCRIPTION

Modification of TestDiet® AIN-93G Semi-Purified Diet, 57W5, with 5% Total Fat, 40% Kcal From Fish Oil, Low Cholesterol & Low Saturated Fat.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1812962

*Other Forms Available On Re

INGREDIENTS (%)

Ingredient	%
Corn Starch	41.8323
Casein - Vitamin Free	20.0000
Maltodextrin	13.2000
Sucrose	10.0000
Powdered Cellulose	5.0000
Fish Oil	4.9163
AIN 93G Mineral Mix	3.5000
AIN 93 Vitamin Mix	1.0000
L-Cystine	0.3000
Choline Bitartrate	0.2500
t-Butylhydroquinone	0.0014

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

2/18/2008

NUTRITIONAL PROFILE [1]

Protein, %	***18.3***
Arginine, %	0.70
Histidine, %	0.52
Isoleucine, %	0.98
Leucine, %	1.73
Lysine, %	1.45
Methionine, %	0.52
Cystine, %	0.37
Phenylalanine, %	0.98
Tyrosine, %	1.01
Threonine, %	0.77
Tryptophan, %	0.22
Valine, %	1.14
Alanine, %	0.55
Aspartic Acid, %	1.29
Glutamic Acid, %	4.08
Glycine, %	0.39
Proline, %	2.38
Serine, %	1.10
Taurine, %	0.00
Fat, %	***5.0***
Cholesterol, ppm	256
Linoleic Acid, %	0.08
Linolenic Acid, %	0.07
Arachidonic Acid, %	0.05
Omega-3 Fatty Acids, %	1.34
Total Saturated Fatty A	1.33
Total Monounsaturated Fatty Acids, %	1.00
Polyunsaturated Fatty Acids, %	1.47
Fiber (max), %	***5.0***
Carbohydrates, %	***65.3***
Energy (kcal/g) [2]	***3.78***

From:	kcal	%
Protein	0.732	19.3
Fat (ether extract)	0.450	11.9
Carbohydrates	2.611	68.8

Minerals	
Calcium, %	0.51
Phosphorus, %	0.32
Phosphorus (available), %	0.16
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.13
Chloride, %	0.22
Fluorine, ppm	1.0
Iron, ppm	36
Zinc, ppm	35
Manganese, ppm	11
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	1.0
Molybdenum, ppm	0.14
Selenium, ppm	0.17

Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	77.5
Vitamin K (as menadione), ppm	0.29
Thiamin Hydrochloride, ppm	6.1
Riboflavin, ppm	6.7
Niacin, ppm	30
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.1
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	29
Choline Chloride, ppm	1,250
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

TestDiet
www.testdiet.com