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# A Small Cohort Omega-3 PUFA Supplement Study: Implications of Stratifying According to Lipid Membrane Incorporation in Cardiac Surgical Patients

Wendy T. K Ip  
*University of Melbourne*

Chanchal Chandramouli  
*University of Melbourne*

Julian A. Smith  
*Monash University*

Peter L. McLennan  
*University of Wollongong, petermcl@uow.edu.au*

Salvatore Pepe  
*University of Melbourne*

*See next page for additional authors*

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# A Small Cohort Omega-3 PUFA Supplement Study: Implications of Stratifying According to Lipid Membrane Incorporation in Cardiac Surgical Patients

## Abstract

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

## Keywords

pufa, omega-3, cohort, small, cardiac, incorporation, membrane, lipid, according, stratifying, implications, patients, study:, surgical, supplement

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## Authors

Wendy T. K Ip, Chanchal Chandramouli, Julian A. Smith, Peter L. McLennan, Salvatore Pepe, and Lea M. D Delbridge

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**A Small Cohort Omega-3 PUFA Supplement Study:  
Implications of Stratifying According to Lipid Membrane  
Incorporation in Cardiac Surgical Patients**

**Wendy T.K. Ip, BBMedSc<sup>a</sup>, Chanchal Chandramouli, BMBSc<sup>a</sup>, Julian A. Smith, FRACS<sup>b</sup>,  
Peter L. McLennan, PhD<sup>c</sup>, Salvatore Pepe, FCSANZ<sup>d\*</sup> and Lea M. D. Delbridge, FCSANZ<sup>a</sup>**

<sup>a</sup> Cardiac Phenomics Laboratory, Department of Physiology, University of Melbourne, Parkville, Australia

<sup>b</sup> Department of Cardiothoracic Surgery, Monash Health, and Department of Surgery, School of Clinical Sciences at Monash Health, Monash University, Melbourne, Australia

<sup>c</sup> Graduate School of Medicine, Centre for Human Applied Physiology, University of Wollongong, Wollongong, Australia

<sup>d</sup> Murdoch Children's Research Institute, Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Melbourne, Australia

**Running Head:**

Omega-3 PUFA & Cardioprotection

**\*Corresponding author**

Assoc.Prof. Salvatore Pepe  
Department of Cardiology  
Royal Children's Hospital  
50 Flemington Road,  
Parkville, VIC 3052, Australia.  
TEL +61-3- 9345 4114  
salvatore.pepe@mcri.edu.au

## **Abstract**

### **Background**

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

### **Methods**

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

### **Results**

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

### **Conclusions**

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

### **Keywords**

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

## Introduction

Evidence of the benefits of dietary intake of omega-3 long chain polyunsaturated fatty acids (n-3 PUFA) first emerged from population studies which identified a potential link between cardiovascular protection and dietary intake of marine fish-derived lipids [1]. Over an extended period, subsequent observational studies also reported positive association between n-3 PUFA consumption and cardiac mortality (although recently concerns have been identified relating to the validity of conclusions drawn from early reports of diet and disease incidence) [2-4]. Beyond observational studies, other investigations have documented anti-arrhythmic effects of n-3 PUFA, delivered via dietary fish inclusion or by fish oil supplementation [5-8].

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

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

The disparity in observational and randomised clinical trial-based findings in relation to n-3 PUFA

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

In this, small cohort, pilot study of patients undergoing elective cardiac surgery, double-blind randomised to receive either n-3 PUFA or placebo, we have explored the association between individual patient tissue lipid status, post-surgical arrhythmia occurrence and levels of selected myocardial molecular measures. Atrial expression of several markers known to be responsive to ischemic stresses, both chronic (characteristic of perfusion insufficiency), and acute (as induced in a surgical setting), were examined. The overall goal was to evaluate the effectiveness of randomization in producing valid, well-contrasted n-3 treatment groups for which treatment effect could be tested by post-hoc measurement of a reliable marker of participant lipid ingestion (erythrocyte membrane lipid composition). The hypothesis was that postoperative arrhythmia suppression and enhanced signalling through pro-survival molecular pathways (ie Bcl, PPAR $\alpha$ ) would be observed in association with n-3 supplement treatment.

## **Materials and Methods**

### **Patient Recruitment**

Patients undergoing elective coronary artery bypass graft (CABG) surgery and/or valve repair/replacement surgery at Monash Medical Centre (Melbourne Australia) were recruited and consented for study participation. The study was approved by the Monash University Human Research Ethics Committee. Exclusion criteria were diabetes diagnosis and absence of stable sinus rhythm at pre-admission check. Patient medication history included ACE inhibition and statin therapy. At pre-admission clinic, patients were randomised to receive either fish oil (FO) or placebo (PO) treatment, 3 capsules/day dispensed by the hospital pharmacy in a double-blinded manner, ongoing for the pre-surgical period. Fish oil capsules (NUMEGA, Clover Corporation, Melbourne Australia) contained 1g tuna fish oil (25% docosahexaenoic acid (DHA, 22:6 n-3), 12% eicosapentaenoic acid (EPA, 20:5 n-3). Placebo capsules were manufactured in parallel and comprised mainly monounsaturated oleic acid (1g Sunola oil). At surgery, right atrial appendage biopsy samples were collected and snap frozen for tissue molecular analysis. Venous blood was collected for analysis of erythrocyte membrane lipids. Evidence of post-operative AF was determined from ECG records obtained during hospitalization and identified as binary (yes/no) occurrence. Thus, three measures were obtained to allow examination of possible relationships between FO supplementation, cell membrane lipid profile and arrhythmia vulnerability post-surgery.

### **Erythrocyte membrane lipid analysis**

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

### Atrial myocardium molecular analyses

To evaluate the proposition that n-3 PUFA supplementation confers myocardial protection in enhancing pro-survival signalling pathways, the levels of several key signalling intermediates known to be responsive to ischemic stress (as induced during surgery) were examined including Bcl2 and Bax (anti- and pro-apoptotic genes), PPAR $\alpha$  (the peroxisomal proliferator-activated receptor, integral to adaptive FA metabolic signalling) and phospho-Akt (the PI<sub>3</sub>-kinase trophic signalling marker).

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

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



were made.

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

### **Statistical analyses**

Data are presented as mean  $\pm$  SEM unless otherwise stated. Differences between groups were assessed using Independent t-test (nominal data, ( $\alpha$ ) of 0.05, ( $\beta$ ) of 0.20) and Pearson chi-square test (categorical data). Regression analysis was performed by linear regression test. Differences were considered significant when  $p < 0.05$ . All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL).

## Results

### Patient PO and FO groups matched for baseline characteristics

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

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

A role for fatty acids in transcriptional regulation has been observed in various settings. Comparing PO and FO treatment groups we sought evidence of n-3 PUFA-associated difference in the expression of key signalling intermediates known to modify myocardial viability and metabolic signalling in ischemic stress. Expression of the apoptosis regulating proteins Bax and Bcl2 at both mRNA and protein levels were similar in the PO and FO groups, evaluated separately and ratiometrically (Figure 1A-C, 1E-G). There were also no differences between PO and FO groups in relation to expression of PPAR $\alpha$  (an energy sensor and regulator molecule) or phosphorylated Akt (a protective intermediate of the PI3Kinase pathway activated by ischemia) (Figure 1D and 1H).

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

### **Correlating expression of cardiac molecular markers with membrane n-3 PUFA content: combining PO and FO measurements**

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

### **Applying an alternative data stratification approach to construct treatment groups**

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

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

Finally, the expression levels of cardiac molecular markers and occurrence of AF was re-evaluated in the treatment groups re-constructed by stratification at the level of 8% total erythrocyte membrane EPA+DHA (Figure 5). With high n-3 PUFA levels (ie  $\geq 8\%$ ), a significant group reduction effect on PPAR $\alpha$  mRNA expression was detected, which had not been evident in the PO vs FO treatment contrast (Figure 5A). For the Bcl2:Bax and for the pAkt:tAkt protein ratios, stratification did not render any difference in outcome when compared with the PO vs FO group analysis (Figure 5B and

5C). In relation to AF, whilst a slightly larger incidence differential was apparent between the stratified groups compared with the randomised groups, this was not a significant finding.

## Discussion

### Analysis approach matters: stratification vs randomisation

Here we report the findings of a small case study (n=20) where cardiac surgery patients were randomized to receive placebo or fish oil, and patient end treatment tissue molecular measures and post-operative AF occurrences were evaluated. Substantial overlap in the level of membrane EPA and DHA in PO and FO groups was observed. Whilst significant correlations between cardiac tissue expression levels of molecular markers involved in myocardial viability and metabolic signalling in ischemia stress and n-3 PUFA content were observed in the pooled patient cohort, no PO or FO group treatment effects could be discerned. Stratification of patient data on the basis of membrane EPA+DHA level (using a threshold value of 8% total membrane lipid) was demonstrated to produce an effective and significant dichotomous separation of patients (by membrane n-6:n-3 ratio). Stratification slightly enhanced the AF group differential compared to randomisation group values, but significant AF benefit of omega-3 status was not detectable in either analysis setting.

### Defining treatment groups in the context of variable omega-3 PUFA background and compliance challenge

The discrepant findings reported from population/observational studies and from RCTs in relation to n-3 PUFA benefit, at least partially reflect the problem of variable participant n-3 PUFA status at study commencement and ongoing ingestion behaviour during the study [15]. In supplementation studies, participants will have variable endogenous background levels of key PUFAs, and the capacity to effect a quantitatively significant intervention by supplementation may be compromised [17]. Evidence suggests that even relatively low dose n-3 PUFA (ie 250mg EPA+DHA/day) can influence cardiovascular outcome, a dose level easily achieved with a single meal dietary adjustment [25, 26]. An early case-control study, at a time before publicity of cardiac benefits of n-3 PUFA, established that erythrocyte EPA+DHA and dietary intake correlated, and both were inversely related to risk of primary cardiac arrest [8]. In the present-day context, study participants with concern of disadvantage conferred by placebo randomisation may self-treat very simply (through independent retail supplement access or dietary modification). Additionally the non-uniform and often short interval from treatment

to surgery can lead to variable and inadequate tissue incorporation required for physiological effect, as demonstrated in animal studies [20, 21]. For future studies it would be desirable to apply exclusion criteria to ensure study participant completion of a minimum period of treatment (ie 28 days as indicated by animal studies) to ensure stabilization of intervention effect on membrane lipid composition.

It has been proposed that an optimal RCT design to detect n-3 PUFA benefit requires selection of study participants with low background n-3 PUFA levels [25-27]. Whilst this approach might have practical/ethical challenge, at least the rigorous implementation of tissue measurement of n-3 PUFA levels at endpoint (and possibly at study entry) seems obligatory. Our membrane analysis data showed clearly that there was substantial overlap in membrane n-3 PUFA content in randomised FO and PO groups. Indeed when stratification was applied (at the erythrocyte 8% EPA+DHA threshold level proposed by von Schacky [25], there was significant group transfer of participants: 20% of the PO group localized into the upper strata, and 44% of the FO participants to the lower strata. The use of the n-6:n-3 PUFA ratio provided particularly useful discrimination between patient groups, and this is consistent with previous clinical and experimental reports [10,28].

### **Myocardial molecular markers, AF and erythrocyte lipids**

Although the end treatment observation of interest in relation to n-3 PUFA supplementation efficacy was post-operative AF, with the retrospective use of stratification it was revealed (post-hoc) that the study as constructed by randomization was not powered to find group differences. An important finding of this study is that the confounding of randomization evidenced by actual membrane lipid composition, limited the capacity to detect group significant difference in AF. In a larger study with increased allocation of participants to the  $\geq 8\%$  stratified group, the power to evaluate group differences in AF occurrence may be increased. Aside from this power limitation, a lack of significant difference in the incidence of AF between group contrasts may also reflect the finding that even the lowest n-3 EPA+DHA content level recorded was  $\sim 6.6\%$ . Recent meta-analysis data indicate that this is not a low value [25], and suggests that all study participants were already potentially amply 'dosed' with n-3 PUFA. Our findings demonstrate that future studies (whether pilot or of more large

scale design) should take into account potential participant baseline fish consumption and that study entry erythrocyte lipid composition should be verified, in order to construct treatment groups with optimal contrast.

By evaluation of pooled data from all patients, negative correlations were found between the content of n-3 PUFA and PPAR levels (and Bcl2:Bax), primarily driven by DHA (compared to EPA). In the stratified data analysis, a significantly reduced PPAR level in the upper level strata ( $\geq 8\%$  total fatty acids) also persisted, whereas Bcl2:Bax findings were marginally not significant. The Bcl2:Bax ratio is generally understood to be an indication of propensity to limit apoptosis. PPAR is known to be responsive to endogenous long chain fatty acids (including n-3 PUFA) and has a role in metabolic signalling through transcriptional regulation of genes [29,30]. The first conclusion is that the apoptotic expression responses appear to be 'early indicators' as the mRNA shifts were not coincident with protein shifts. Secondly, the finding of inverse correlation between n-3 PUFA membrane content and both PPAR and Bcl2:Bax mRNA ratio is suggestive of n-3 PUFA signalling feedback inhibition. Although not extensive, there is some experimental literature suggesting n-3 PUFA apoptosis suppression in oncogenic settings and neural cell types [31,32]. However as we did not directly investigate a panel of protein apoptotic measures (due to specimen size constraints), our findings in this study cannot be considered mechanistically definitive but do provide a basis for further discovery work in larger treatment groups in relation to n-3 transcriptional and translational regulation in the heart. Despite the limitations of measuring only a small number of 'candidate' genes and proteins, and the constrained group sizes which emerged after data stratification, these molecular markers provided utility for exploring secondary end treatment observations .

## **Conclusion**

The major finding of this small n-3 PUFA case study is the demonstration that the same cohort may yield differing findings when evaluated using randomisation or stratification approaches based on direct molecular measurements of cell membrane lipids. In this pilot study a significant finding through stratification in relation to differential cardiac expression of the PPAR $\alpha$  was determined. In an expanded study using stratification measures, a definitive finding in relation to n-3 supplementation and AF occurrence may be achievable.

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## **Disclosures**

None.



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

Treatment	Patient	Age	Gender	Treatment period	Post-surgical AF occurrence
Placebo	M73	66	M	15	N
Placebo	M74	67	M	14	N
Placebo	M77	79	M	40	Y
Placebo	M80	65	M	5	N
Placebo	M81	73	M	44	Y
Placebo	M86	78	M	12	Y
Placebo	M89	53	M	5	N
Placebo	M90	70	M	16	Y
Placebo	M76	55	F	36	Y
Placebo	M84	65	F	7	N
Mean PO		67 $\pm$ 3 (years)	80% M (8M + 2F)	19 $\pm$ 5 (days)	50% (5/10)
Fish oil	M71	55	M	9	N
Fish oil	M72	66	M	11	N
Fish oil	M75	69	M	12	N
Fish oil	M79	73	M	18	Y
Fish oil	M83	53	M	12	N
Fish oil	M85	44	M	4	N
Fish oil	M87	71	M	22	Y
Fish oil	M78	36	F	108	N
Fish oil	M82	73	F	6	Y
Fish oil	M88	70	F	7	N
Mean FO		61 $\pm$ 4 (years)	70% M (7M + 3F)	21 $\pm$ 10 (days)	30% (3/10)

## Figures

### Figure 1. Cardiac molecular markers in PO and FO groups.

**A-D.** mRNA expression (by qRT-PCR) of apoptotic proteins Bax and Bcl2, and metabolic transcriptional factor PPAR.

**E-H.** Protein expression (by immunoblot) of apoptotic proteins Bax and Bcl2, and phospho-activation of trophic mediator Akt (Ser 473) normalized to total Akt (pAkt:tAkt).

Data presented mean  $\pm$  SEM. Non-paired t-test,  $p = n.s.$  PO, Placebo Oil; FO, Fish Oil.

### Figure 2. Membrane n-3 content vs treatment period and age

**A, B.** Regression analysis of erythrocyte membrane EPA+DHA and DHA only (% total fatty acids) with patient treatment duration in PO and FO groups.

**C, D.** Regression analysis of erythrocyte membrane EPA+DHA and DHA only (% total fatty acids) with patient age in PO and FO groups.

Data analysed by linear regression test. PO, Placebo Oil; FO, Fish Oil.

### Figure 3. Membrane n-3 content vs cardiac molecular marker expression

**A.** Correlation of cardiac molecular markers with erythrocyte EPA content (pooled PO & FO groups).

**B.** Correlation of cardiac molecular markers with erythrocyte DHA content (pooled PO & FO groups).

**C.** Correlation of cardiac molecular markers with erythrocyte total EPA+DHA (pooled PO & FO groups).

Data analysed by linear regression test.  $n = 11-16$ ;  $*p < 0.05$ . EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

**Figure 4. Membrane EPA+DHA: groups by randomisation and stratification**

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

**B.** Erythrocyte membrane EPA+DHA as % fatty acids for < 8% and  $\geq$  8% stratified groups

*Data shown as dot plots with mean (horizontal bar), and threshold stratification level (8%) dashed line.*

**C.** Erythrocyte membrane n-6:n-3 ratio for randomized and stratified groups.

*Data presented mean  $\pm$  SEM. Non-paired t-test, \* $p < 0.05$ .*

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

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

**A.** PPAR mRNA expression for randomised and stratified groups.

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

**C.** pAkt (ser 473):tAkt protein expression ratio for randomized and stratified groups.

*Data presented mean  $\pm$  SEM. Non-paired t-test, \* $p < 0.05$ .*

**D.** AF occurrence for randomised and stratified groups.

*For stratified groups membrane EPA+DHA as % fatty acids (< 8%,  $\geq$  8% total fatty acids). Data analysed by Pearson Chi-square test  $p = n.s.$*

*PO, Placebo Oil; FO, Fish Oil.*

**Figure 4. Membrane EPA+DHA: groups by randomisation and stratification**

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

**B.** Erythrocyte membrane EPA+DHA as % fatty acids for < 8% and  $\geq$  8% stratified groups

*Data shown as dot plots with mean (horizontal bar), and threshold stratification level (8%) dashed line.*

**C.** Erythrocyte membrane n-6:n-3 ratio for randomized and stratified groups.

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*PO, Placebo Oil; FO, Fish Oil.*

Figure 1.

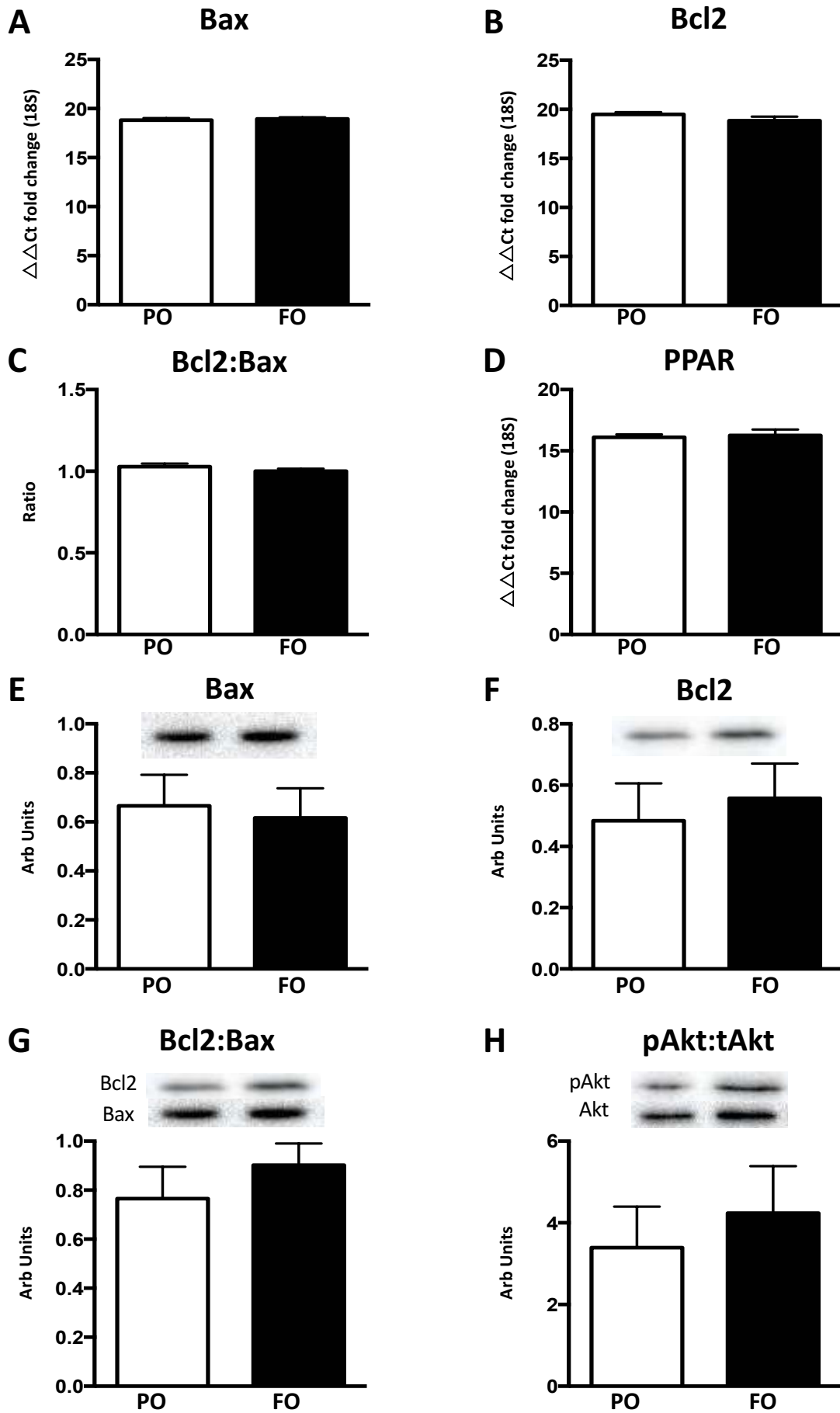
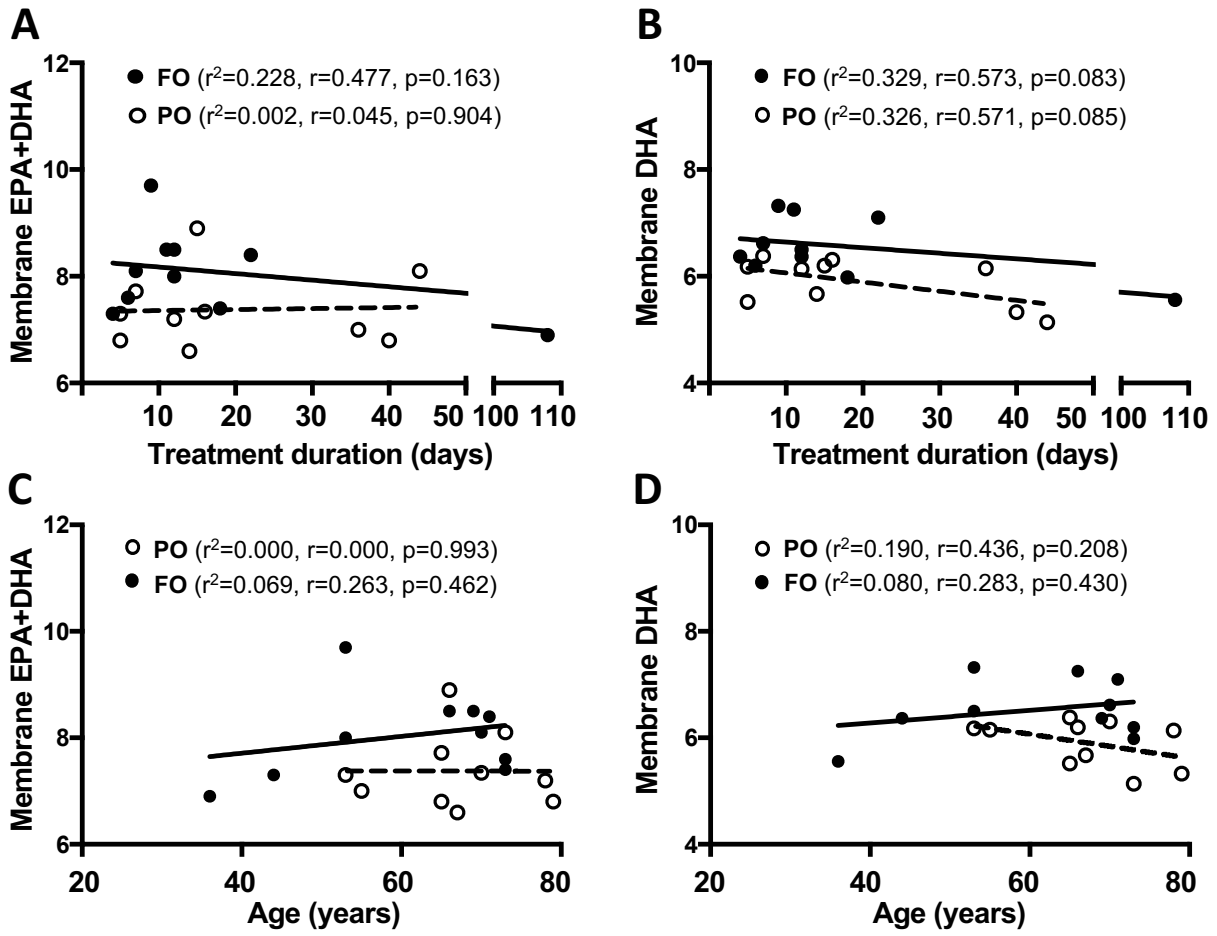


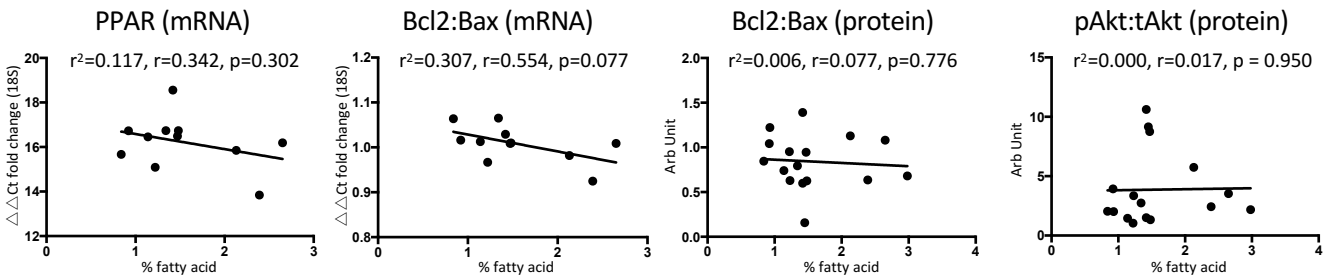


Figure 2.

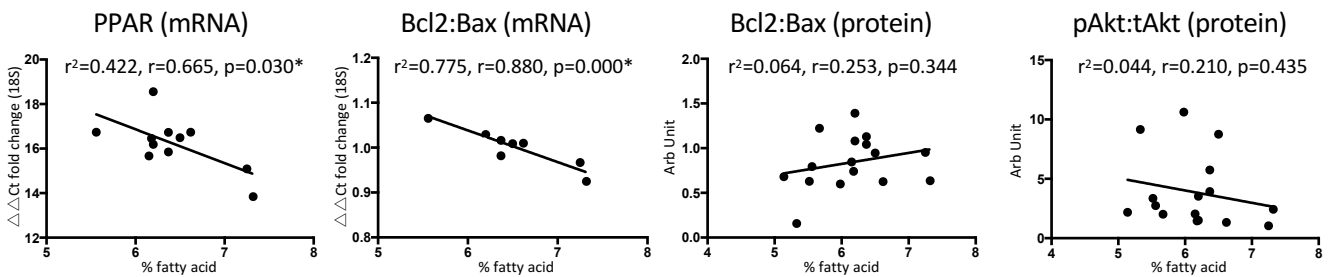


**Figure 3.**

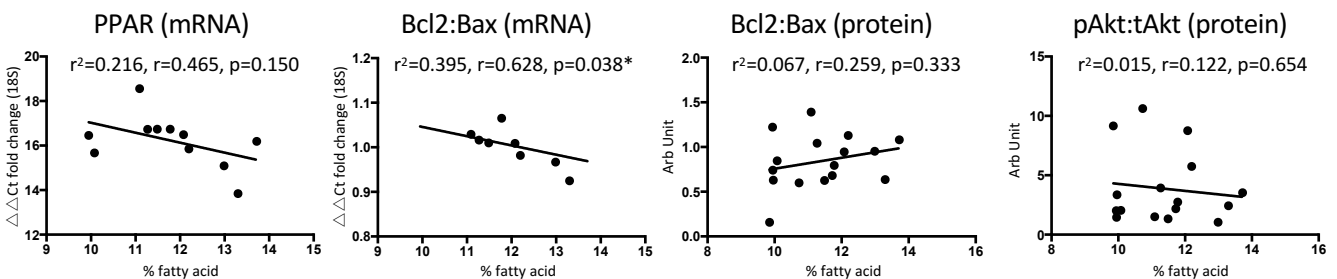
**A EPA**



**B DHA**



**C Total n-3 PUFA**



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

Figure 4.

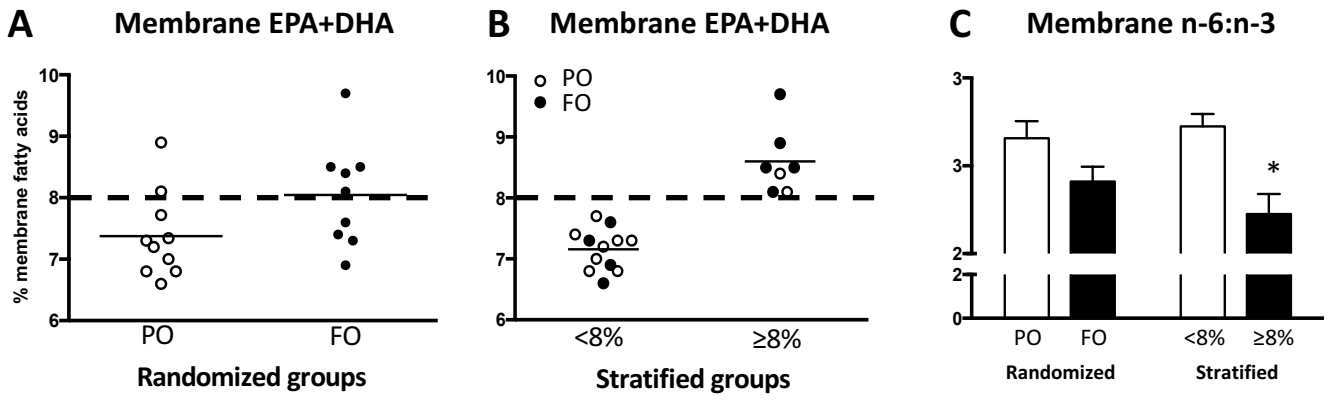


Figure 5.

