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n-3 PUFAs improve erythrocyte fatty acid profile in patients with small abdominal aortic aneurysms: a randomised controlled trial

Lara T. Meital[1,2], Mark T. Windsor[2], Rebecca M.L. Ramirez Jewell[1], Peter Young[3], Karl Schulze[4], Rebecca Magee[5], Jill O'Donnell[5], Pankaj Jha[5], Maria Perissiou[2], Jonathan Golledge[6,7], Tom G. Bailey[2,8], Peter Brooks[1], Christopher D. Askew[2], Fraser D. Russell[1,2]

[1] *GeneCology Research Centre, University of the Sunshine Coast, Qld, Australia.*

[2] *VasoActive Research Group, School of Health and Sport Sciences, University of the Sunshine Coast, Qld, Australia.*

[3] *Technical Services, University of the Sunshine Coast, Qld, Australia.*

[4] *Sunshine Vascular, Buderim, Qld, Australia*

[5] *Sunshine Coast University Hospital, Birtinya, Qld, Australia*

[6] *Queensland Research Centre for Peripheral Vascular Disease, College of Medicine and Dentistry, James Cook University, Townsville, Australia*

[7] *Department of Vascular and Endovascular Surgery, Townsville Hospital, Townsville, Australia*

[8] *Centre for Research on Exercise, Physical Activity and Health, School of Human Movement and Nutrition Sciences, The University of Queensland, Qld, Australia*

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Corresponding author:

F.D. Russell

Genecology Research Centre,

School of Health and Sport Sciences,

University of the Sunshine Coast, Maroochydore, QLD 4558, Australia.

Tel: +61 7 5459 4665

Email: frussell@usc.edu.au

Abbreviations: abdominal aortic aneurysm, AAA; omega-3 polyunsaturated fatty acids, n-3 PUFAs; arachidonic acid, AA; red blood cell distribution width, RDW;

Abstract

Abdominal aortic aneurysm (AAA) is an important cause of death in older adults which has no current drug therapy. Inflammation and abnormal redox status are believed to be key pathogenic mechanisms for AAA. In light of evidence correlating inflammation with aberrant fatty acid profiles, this study compared erythrocyte fatty acid content in 43 AAA patients (diameter 3.0-4.5 cm) and 52 healthy controls. In addition, the effect of omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation on erythrocyte fatty acid content was examined in a cohort of 30 AAA patients as part of a 12-week randomised placebo-controlled clinical trial. Blood analyses identified associations between AAA and decreased linoleic acid, and AAA and increased Δ6-desaturase activity and biosynthesis of arachidonic acid from linoleic acid. n-3 PUFA supplementation (1.5g DHA+0.3g EPA/day) decreased red blood cell distribution width (RDW, $14.8 \pm 0.4\%$ to $13.8 \pm 0.2\%$, p=0.003) and levels of pro-inflammatory n-6 PUFAs (arachidonic acid, $12.46 \pm 0.23\%$ to $10.14 \pm 0.3\%$, P<0.001; adrenic acid $2.12 \pm 0.13\%$ to $1.23 \pm 0.09\%$, p<0.001). In addition, Δ-4 desaturase activity increased (docosahexaenoic/docosapentaenoic acid ratio, 1.85 ± 0.14 to 3.93 ± 0.17, P<0.001) and elongase 2/5 activity decreased (adrenic acid/arachidonic acid ratio, 0.17 ± 0.01 to 0.12 ± 0.01, P<0.01) following supplementation. The findings suggest n-3 PUFAs improve fatty acid profiles and ameliorate factors associated with inflammation in AAA patients.

Keywords: omega-3 fatty acids, antioxidants, clinical studies, diet and dietary lipids, diet effects/lipid metabolism, abdominal aortic aneurysm, omega-3 index

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Introduction

Abdominal aortic aneurysm (AAA) is a full-thickness dilation of the infrarenal aorta that can lead to lethal artery rupture (1). The global prevalence of AAA in adults over the age of 65 y is estimated to be between 1 and 3% (2). While chronic aortic inflammation, oxidative stress and enhanced proteolytic enzyme activity are implicated in the destructive remodelling of aortic connective tissue that occurs in AAA, (3, 4), no recognised drug therapies exist to halt or reverse these aberrant molecular processes or their injurious mechanical sequelae.

Technical advances in mass spectrometry methods have resulted in the availability of tools to accurately profile the human lipidome (5) and, more specifically, quantify the accumulation of omega-3 polyunsaturated fatty acids (n-3 PUFAs) in red blood cell membranes. Animal data have suggested the potential of n-3 PUFA supplementation to limit small AAA growth through positive impacts on oxidative stress status (6, 7) and interference with inflammatory cascades (8-10). While clinical evidence in AAA patients is limited, low serum eicosapentaenoic acid (EPA) levels have been reported to be associated with larger aneurysm size and faster growth rate in a Japanese patient population (11). A separate study involving a Danish cohort of patients, however, found no association between n-3 PUFA blood status and the development or growth of AAA (12). n-3 PUFA blood status is typically evaluated by omega-3 index measurement (13). The omega-3 index represents the ratio of EPA + docosahexaenoic acid (DHA) to all other fatty acids incorporated in red blood cell membrane phospholipids and substantial published research suggests that this measurement is a reliable predictor of cardiovascular event risk (14-17). In light of this, and evidence correlating specific disease states with distinct fatty acid profiles (18-20), the present study was designed to compare full blood count data, baseline omega-3 index values and fatty acid profiles of erythrocytes obtained from AAA patients with those of a healthy control cohort as part of an ongoing clinical trial. In addition, the impact of physiologically appropriate n-3 PUFA supplementation on full blood count values, the omega-3 index and erythrocyte membrane fatty acid composition was examined in a cohort of AAA patients as part of a 12-week randomised, double-blind, placebo-

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controlled clinical trial. It was hypothesised that comparative analysis of erythrocyte fatty acid composition would yield a distinct AAA fatty acid profile that could direct future progress toward identification of novel therapeutic targets.

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Materials and Methods

Observational (case-control) study

AAA patients were recruited from the Sunshine Coast University Hospital and a private clinic (Sunshine Vascular) and healthy control participants were recruited from the general population of the Sunshine Coast, Queensland with approval from the University of the Sunshine Coast (A/13/473 and A/16/833) and the Prince Charles Hospital Human Research Ethics Committees (HREC/16/QPCH/114 and HREC/12/QPCH/13). The patient group included 43 men with small AAA (diameter 3.0-4.5 cm) and the control group included 52 men without a documented AAA. It has previously been reported that there are significant differences in omega-3 index between men and women (21), and AAA is known to be much more common in men than women (22). In view of this, only men were included in the current study. Written informed consent was obtained for each participant. Maximum AAA diameter was measured by ultrasound prior to study entry. Exclusion criteria included age below 60 or above 86 years, body mass index (BMI) above 39 kg·m^{-2}, uncontrolled hypertension, cardiac arrhythmia, heart failure, symptomatic aortic stenosis, chronic obstructive pulmonary disease, chronic inflammatory disease and regular use of prescription anti-inflammatory medication. A family history of AAA or known aneurysmal disease served as additional exclusion criteria for control participants. All participants refrained from non-prescribed anti-inflammatory medications 72 h prior to blood collection and abstained from alcohol and caffeine for the 12 h leading up to their study visit.

Omega-3 clinical trial

The impact of n-3 PUFA supplementation on erythrocyte fatty acid composition was investigated in AAA patients as part of a parallel-design, double-blind, placebo-controlled trial (ANZCTR12616000483459), carried out between June 2017 and June 2018. Patients with small (3.0 - 4.9 cm) AAA (n=32) were recruited from Nambour General Hospital, Sunshine

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Coast University Hospital and a private clinic (Sunshine Vascular) and randomised (Figure 1) to receive either 1) active fatty acids (3 Blackmores Omega Brain capsules each containing 500 mg DHA and 100 mg EPA and delivering a total of 1.8 g n-3 PUFAs per day) or 2) placebo fatty acids (3 capsules each containing 490 mg corn oil, 490 mg olive oil and 20 mg fish oil) for a period of 12 weeks. Capsules were of similar appearance and flavour. Participants were supplied with capsules at day 0, week 3 and week 8, with instruction to take three each morning. The final study visit was at week 12. A substantial research literature suggests the intervention dose and duration selected for the clinical trial will be sufficient to raise the omega-3 index of supplemented subjects to protective levels associated with positive cardiovascular outcomes (23, 24). Exclusion criteria for the clinical trial were: age below 55 or above 86 years, consumption of three or more fish meals per week, the use of fish oil or krill oil supplements and the use of anti-inflammatory medications. Written informed consent was obtained from each participant and information regarding medical history, physical activity and dietary intake of n-3 PUFAs was collected. The protocol was approved by the University of the Sunshine Coast (Ethics Approval Number A/16/833) and the Prince Charles Hospital Human Research Ethics Committees (HREC/16/QPCH/114). The study was conducted in accordance with the Declaration of Helsinki (1964).

Randomisation and evaluation of compliance

An Excel block randomisation algorithm was used to assign participants to either an active or a placebo treatment protocol. Random selection of block size 4 or 6 during the computerised sort avoided selection bias (25). Sequentially numbered containers were used to implement the random allocation of capsules. The randomisation allocation sequence was generated by the corresponding author. Compliance with treatment protocols was evaluated by monitoring capsule returns and by gas chromatography-mass spectrometry (GC-MS) measurement of red blood cell omega-3 index.

Blood sample collection

Fasting blood samples were collected from AAA patients and healthy control participants into EDTA-containing tubes and serum separator tubes. Erythrocytes from EDTA tubes were sedimented by centrifugation (1500 x g, 15 min, 15°C), the plasma and buffy coat fractions were removed and the packed red blood cells were stored at -80°C until analysis. Blood collected into serum separator tubes was allowed to clot at 22°C for 30 min prior to centrifugation (1500 x g, 15 min, 15°C). The serum was collected and centrifuged (4000 x g, 5 min, 4°C) and the supernatant was stored at -80°C until processing and analysis. For the omega-3 clinical trial, fasted blood samples were collected from AAA patients at day 0 (prior to initiation of the study), at day 21 (following 3 weeks of supplementation) and at day 84 (following 12 weeks of supplementation). Erythrocytes from EDTA tubes were processed and stored as described above.

Erythrocyte fatty acid analysis

Choice of erythrocytes as the preferred matrix for assessment of n-3 PUFA status was based on evidence suggesting 1) measurement of erythrocyte fatty acid levels assesses long-term dietary fatty acid intake, 2) erythrocyte fatty acid levels are less sensitive than plasma to day-to-day n-3 PUFA intake, 3) erythrocyte n-3 PUFA levels display one-fourth of the within-person variability that occurs with plasma and 4) erythrocyte n-3 PUFA levels are highly correlated with those in a variety of tissues (26). Erythrocyte fatty acid composition was determined using GC-MS as previously described (27). Briefly, a 600 µl aliquot of methanol containing butylated hydroxytoluene (BHT; 20 mg/100 ml) as an antioxidant was added to erythrocyte samples (300 µl). The cells were homogenised with glass rods (1 min), flushed with nitrogen gas and incubated on ice for 30 min. A 600 µl aliquot of chloroform was added to the suspension and cells were homogenised, flushed and incubated as before. The preparation was centrifuged (3000 x g, 4°C, 5 min) and the supernatant was withdrawn, flushed with nitrogen gas and stored on ice. This procedure was repeated twice with 300 µl volumes of methanol/BHT and

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chloroform and incubation periods of 10 min. In a final extraction step, 1 ml of pooled lipid supernatant was combined with chloroform (800 μl) and KCl (0.05M; 460 μl), the solution was mixed by vortex, flushed with nitrogen gas and centrifuged (3000 x g, 4°C, 10 min). The supernatant was discarded and the fraction containing lipids was dried under nitrogen gas. The extracted lipids were hydrolysed in the presence of 500 μl of 9M HCl:H_2O:acetonitrile (1:1:18 containing 25 mg/50 ml BHT) during overnight incubation at 65°C. The hydrolysed samples were dried under nitrogen gas and, following a 10 min incubation at -80°C, were freeze-dried (Thermo Savant Modulyo Freeze Dryer System, Thermo Fisher Scientific) for 30 min. Samples were derivatised in 250 μl of hexane containing 10 μl 1-tert-butyldimethylsilylimidazole during a 2h incubation at 37°C. Fatty acids were separated and analysed with a Perkin Elmer Clarus 580 gas chromatograph coupled to a Perkin Elmer Clarus SQ85 mass spectrometer using a Perkin Elmer Elite 5MS column (30m x 0.25 mm ID x 0.25 mm film). The GC method included a split ratio of 30:1 with injector at 300°C and temperature programming of 170°C (initial) ramped to 310°C at a rate of 6°C/min followed by a 5 min hold period. The MS method included ionisation at 70 eV with a scan range of 45-450 m/z for 4.0-28.3 min. Peaks were identified by comparison with known standards or the National Institute of Standard and Technology Library (NIST 2008 Library). Omega-3 indices were expressed as the combined percentage of DHA and EPA integrated peak areas divided by the total fatty acid integrated peak areas.

Enzyme activity

Product to precursor erythrocyte phospholipid fatty acid ratios served as indices of activity levels of enzymes involved in fatty acid metabolism. The index of Δ-9 desaturase activity was calculated as the ratio of oleic acid to stearic acid (28) and the index of Δ-4 desaturase activity was calculated as the ratio of docosahexaenoic acid (DHA) to docosapentaenoic acid (DPA) (29). The index of Δ-6 desaturase was calculated in two ways a) as a ratio of dihomo-γ-linolenic acid to linoleic acid (29) (this calculation includes the activities of the enzymes Δ-6

desaturase and elongase 5) and b) as a ratio of γ-linolenic acid to linoleic acid (28) (this calculation includes the activity of Δ-6 desaturase only). The ratio of arachidonic acid (AA) to linoleic acid (LA) served as an index of desaturase/elongase-mediated LA $\rightarrow$ AA biosynthesis (30). The index of stearoyl-CoA desaturase activity was calculated as the ratio of palmitoleic acid to palmitic acid (29) and the index of elongase 2/5 activity was calculated as the ratio of adrenic acid to AA (31).

Full blood count analysis

Whole blood from fasted participants was collected into EDTA tubes and full blood count analyses were conducted within 10 min of the collection using a Coulter $A^C \cdot T$ diff™. Data regarding red blood cell indices (haemoglobin, HgB; haematocrit, Hct; mean corpuscular volume, MCV; mean corpuscular haemoglobin, MCH; mean corpuscular haemoglobin concentration, MCHC; red blood cell distribution width, RDW), white blood cell counts (lymphocytes, monocytes, granulocytes) and platelet counts and indices (mean platelet volume, MPV; plateletcrit, Pct; platelet distribution width, PDW) were collected. Instrument calibration was verified prior to measurement of each sample using appropriate quality controls.

Data analysis

Observational (case-control) study

Erythrocyte fatty acid content and full blood count analysis have not been investigated previously in small AAA and healthy control participants. Group size estimates for the observational study were based on RDW values reported in healthy controls (RDW, 13.1±1.3, n=40) and patients with coronary artery ectasia (RDW, 14.8±1.6, n=54) (32). A group size estimate of 14 was calculated with 85% power (alpha level of 0.05) using power/sample size (Univ. British Columbia) and pooled variance (Solvers statistics) calculators.

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Omega-3 clinical trial

The group size estimate for the omega-3 clinical trial was based on fatty acid values obtained from a 5-month omega-3 fatty acid supplementation trial (1.8 g/day) in a healthy cohort (33). Baseline erythrocyte levels (n=23) were: arachidonic acid (16.1±1.2%), adrenic acid (4.1±0.5%), EPA (0.5±0.4%) and DHA (3.9±0.8%). End-of-study levels (n=24) were: arachidonic acid (13.6±1.2%), adrenic acid (2.6±0.5%), EPA (2.5±0.4%) and DHA (7.0±0.8%). Group size estimates were between 1 and 4 (85% power; alpha level of 0.05).

Continuous demographic data for AAA patients and control participants were compared using a student's t-test and are presented as mean±SD. Categorical demographic variables were compared using a Fisher's exact test. Experimental data are presented as mean±SEM and between group differences were examined by student's t-test analysis. The association between independent variables identified as being significantly different (see Table 8) and AAA was assessed using linear regression analysis with adjustment for covariates shown to be imbalanced between groups (hypertension, diabetes mellitus, coronary heart disease, dyslipidaemia, use of statins or low-dose aspirin, current and previous smoking history, and age). All variables were introduced in one step in order of decreasing tolerance. Data were analysed with Prism (GraphPad Software, La Jolla, CA). IBM SPSS Statistics Version 24 was used for multi-variable regression analysis and statistical significance was set at p<0.05.

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Results

Observational (case-control) study

Baseline characteristics

Baseline characteristics of the case-control study are shown in Table 1. AAA patients were older and were characterised by a higher prevalence of hypertension, diabetes mellitus, dyslipidaemia, CHD and smoking history. AAA patients were more commonly prescribed statins and anti-platelet drugs.

Fatty acid methyl ester analysis

Twenty-three fatty acid methyl esters were consistently identified in erythrocyte membranes obtained from AAA patients and healthy control participants (Supplemental Table S1).

Erythrocyte fatty acid profiles

Mean omega-3 index values were similar in cases and controls while the mean omega-6:omega-3 (n-6/n-3) ratio was significantly lower than the corresponding value obtained for the control cohort (p=0.047; Table 2). Levels of the saturated fatty acid margaric acid (C17:0) were significantly higher in AAA patients compared to healthy control participants (p=0.007) while levels of the n-6 fatty acid, linoleic acid (C18:2) were significantly lower (p=0.007). The n-3 PUFA, DPA (C22:5) was significantly higher in erythrocytes from AAA patients compared to control participants (p<0.001). Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age (Table 8).

Enzyme activity

The indices of Δ-4 (DPA→DHA) and Δ-9 desaturase (stearic→oleic) activity in erythrocytes from AAA patients were comparable to the control cohort (Table 3) as were the index of stearoyl-CoA desaturase (palmitic→palmitoleic acid) activity and the index of elongase 2/5 (AA→adrenic acid) activity. The index of Δ-6 desaturase (1) activity was significantly higher in erythrocytes from AAA patients compared to control participants (p=0.005), as was the index of desaturase/elongase-mediated LA → AA biosynthesis (p=0.008). Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age (Table 8).

Red blood cell indices, white blood cell counts and platelet counts and indices

Red blood cell distribution width and other red blood cell indices were similar in cases and controls, as were white blood cell counts and platelet counts and indices following adjustment for covariates shown to be imbalanced between groups (Table 4).

Omega-3 clinical trial

Baseline characteristics

Baseline characteristics were similar between groups with the exception of statin use (Table 1).

Participant compliance and tolerability

A high level of adherence to supplement intake was identified by return capsule counts (Placebo: 96.4±1.2, 96.8±2.1, and 95.0±2.9% at Weeks 3, 8 and 12, respectively; omega-3 fatty acid group: 97.2±0.9%, 94.0±1.7% and 95.0±1.6% at Weeks 3, 8 and 12, respectively). This was supported by GC-MS analysis of fatty acid incorporation in red blood cell membrane phospholipids. The omega-3 index was markedly increased in all participants in the omega-3 fatty acid group over the 12-week trial period (Table 5). No change in omega-3 index was observed in participants who were randomised to receive placebo capsules. Two participants

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in the placebo group withdrew from the trial citing gastrointestinal disturbances (Figure 1). Among participants who completed the 12-week trial, three in the placebo group reported burping, with one of these also experiencing nausea. In the omega-3 fatty acid group, three participants reported burping or reflux, and one participant reported flatulence.

Erythrocyte fatty acid profiles in AAA patients following n-3 PUFA supplementation

Significant increases in DHA (76%, p<0.001) and EPA (69%, p<0.001) levels were observed in erythrocytes from AAA patients following 12 weeks of n-3 PUFA supplementation (Table 5). This effect was accompanied by significant decreases in the n-6 fatty acids arachidonic acid (AA, C20:4, p<0.001), adrenic acid (C22:4, p<0.001) and dihomo-γ-linolenic acid (C20:3, p=0.003) from baseline to week 12. Additional fatty acids altered by n-3 PUFA supplementation included the n-3 PUFA docosapentaenoic acid (DPA, C22:5) and the very long chain fatty acid nervonic acid (C24:1), both of which decreased at week 12 (p=0.004 and p=0.009 respectively). The mean omega-3 index value among AAA patients increased at week 3 (33%, p<0.001) and week 12 (74%, p<0.001) while the mean n-6/n-3 ratio decreased at these time points (22%, p<0.001 and 42%, p<0.001 respectively). No changes in any fatty acid levels were observed for the placebo group from baseline to week 3 or from baseline to week 12.

Enzyme activity

Twelve-week n-3 PUFA supplementation significantly increased the index of Δ-4 desaturase (DPA→DHA) activity in erythrocytes from AAA patients (p<0.001) while the index of Δ-6 desaturase (LA→DGLA and LA→GLA) activity, the index of Δ-9 desaturase (stearic→oleic) activity and the index of stearoyl-CoA desaturase (palmitic → palmitoleic acid) activity remained unchanged (Table 6). Omega-3 PUFA supplementation of AAA patients lowered the index of desaturase/elongase-mediated LA → AA biosynthesis and the index of elongase 2/5 (AA→adrenic acid) activity to levels that were comparable to the control cohort (p=0.031 and

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p=0.002 respectively). No changes in any enzyme activity levels were observed for the placebo cohort.

Omega-3 fatty acid supplementation decreases red blood cell distribution width

Significant decreases in red blood cell distribution width (p=0.003), red blood cell counts (p=0.019), haematocrit percentage (p=0.016), platelet counts (p=0.042) and plateletcrit percentage (p=0.021) were observed for AAA patients following 12 weeks of n-3 PUFA supplementation (Table 7).

Discussion

Observational (case-control) study

Analysis of erythrocyte fatty acid profiles among AAA patients highlighted a mean omega-3 index value that was comparable to the control cohort in conjunction with an n-6/n-3 fatty acid ratio that was significantly lower. Among individual fatty acids measured, levels of the saturated fatty acid margaric acid and the omega-3 fatty acid DPA were significantly higher in erythrocytes from AAA patients and levels of the omega-6 fatty acid linoleic acid were significantly lower compared to control. The lower levels of linoleic acid in conjunction with higher levels of adrenic acid in the AAA cohort are of note. A meta-analysis of prospective cohort studies (n=310,602 participants, n=12,479 cases) indicated that highest levels of dietary linoleic acid intake were associated with a 15% lower risk of CHD events and a 21% lower risk of CHD deaths when compared to lowest levels of linoleic acid intake (34). In addition, a higher level of circulating linoleic acid in a community-based US cohort (n=2,792) was associated with lower total mortality risk (extreme-quintile hazard ratio=0.87; P=0.005) and a lower risk of cardiovascular disease mortality (22% lower risk in the highest versus the lowest quintile; P=0.02) (35). It has been suggested that the observed cardio-protective effects of linoleic acid are related to its competition with pro-inflammatory arachidonic acid for re-acylation into membrane phospholipids (36). In line with this, a strong inverse association has been reported between serum linoleic acid levels and high-sensitivity C-reactive protein (hsCRP), a key marker of inflammation (37). These findings suggest that the altered n-6 fatty acid profile in AAA skews cellular responses toward pro-inflammatory eicosanoid production and upregulated inflammatory pathways.

Desaturase enzymes catalyse the rate-limiting steps in long chain fatty acid synthetic pathways and their activities influence erythrocyte phospholipid fatty acid composition (28). In this study, Δ-4 and Δ-9 desaturase activity levels in erythrocytes from AAA patients were comparable to those of a healthy control cohort. In contrast, the index of desaturase/elongase-

mediated LA → AA biosynthesis was significantly higher in erythrocytes from AAA patients compared to control participants, as was the index of Δ-6 desaturase activity. The higher index of Δ-6 desaturase activity suggests anti-inflammatory n-3 PUFA formation is repressed in favour of pro-inflammatory n-6 PUFA biosynthesis. It is of note that the higher index of desaturase/elongase-mediated LA → AA biosynthesis did not translate into higher levels of arachidonic acid in this cohort. Arachidonic acid is a known substrate for inflammatory eicosanoid synthesis and, while it is possible that the absence of an increase in arachidonic acid levels in AAA patients may be due to excessive shunting of this fatty acid to an alternative eicosanoid biosynthetic pathway, we have previously reported lower levels of prostaglandin E_2 (a product of arachidonic acid metabolism) in this AAA cohort compared to healthy control participants. It is thus possible that the higher conversion of LA to AA in AAA patients led to increased flux through the n-6 biosynthetic pathway beyond arachidonic acid to adrenic acid resulting in the observed higher levels of the latter. The anomalies observed in enzyme activity indices among AAA patients suggest a predisposition toward reactions that favour increased production of n-6 PUFAs with high pro-inflammatory potential and repressed production of their anti-inflammatory n-3 PUFA counterparts.

Omega-3 clinical trial

Twelve-week n-3 PUFA supplementation decreased levels of the n-6 fatty acids arachidonic acid and adrenic acid and increased levels of the n-3 fatty acids DHA and EPA in AAA patient erythrocytes while concomitantly raising the mean omega-3 index to a value almost double that at study entry (8.03%). Importantly, the post-supplementation omega-3 index value was within the 8-12% range that affords cardioprotection (26). The large reduction in arachidonic and adrenic acid levels with n-3 PUFA supplementation likely reflects competition of DHA and EPA for incorporation into existing erythrocyte membranes and/or greater availability of n-3 PUFAs for integration into newly synthesised membranes (38). The anti-inflammatory and immunomodulatory properties of increased cellular phospholipid EPA and DHA levels are likely to favourably impact AAA disease. Higher n-3 PUFA intake results in partial

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substitution of arachidonic acid for EPA and DHA, a net decrease in pro-inflammatory eicosanoid production and favourable impacts on inflammatory responses (39). The latter is due to competition of n-3 PUFAs with arachidonic acid for the same metabolic enzymes resulting in production of an alternate series of less biologically potent eicosanoids with weaker pro-inflammatory, platelet-aggregating and vasoconstrictive activities (39-41). Omega-3 PUFAs are, in addition, substrates of cytochrome P450 enzymes and increasing the levels of these fatty acids results in enhanced production of DHA- and EPA-derived metabolites at the expense of arachidonic acid-derived metabolite production (42). The lower n-6/n-3 ratio, a value reflecting the balance between precursor PUFAs giving rise to downstream pro- and anti-inflammatory eicosanoids respectively, supports an n-3 PUFA-driven switch towards a more favourable eicosanoid profile.

Twelve-week n-3 PUFA supplementation significantly increased the index of Δ-4 desaturase activity in erythrocytes from AAA patients and lowered the index of desaturase/elongase-mediated LA → AA biosynthesis and the index of elongase 2/5 activity to levels that were comparable to the control cohort (Supplemental Figure S1). The index of Δ-6 desaturase activity was unaffected by n-3 PUFA supplementation. Δ-4 Desaturase and Δ-6 desaturase each form part of a distinct biochemical pathway that yields DHA as a biosynthetic product. Δ-6 Desaturase forms part of a coupled microsomal-peroxisomal pathway that produces DHA through sequential desaturations and elongations, while Δ-4 desaturase forms part of an alternative pathway that yields DHA through a single Δ4 desaturation step (43). As expected with DHA supplementation, the ratio of DHA:DPA increased. Determination of the contribution of Δ-4 desaturase, if any, to this result would require direct measurement of the activity of this enzyme. The decreases in enzyme activities leading to pro-inflammatory n-6 PUFA production suggest n-3 PUFA supplementation alters fatty acid metabolism in a manner that is likely to improve the inflammatory status of AAA patients.

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Twelve-week supplementation with n-3 PUFAs (1.8g/d) significantly decreased RDW, red blood cell counts, haematocrit percentage and platelet count and platelet percentage in AAA patients. RDW is a numerical measure reflecting size variability or heterogeneity of volume among circulating erythrocytes (44). It is well documented that RDW correlates with inflammatory biomarkers in a multitude of clinical settings (45-47) and a large cohort study has supported the existence of a strong graded relationship between RDW and high sensitivity C-Reactive Protein and RDW and erythrocyte sedimentation rate, independent of confounding factors (48). In light of this and evidence suggesting the existence of extensive cross-talk between the pathways of inflammation and coagulation (49, 50), it is likely that the observed changes reflect n-3 PUFA-mediated improvements in inflammatory status in AAA patients who received this supplement.

The study, while limited by small sample size, was characterised by multiple strengths that included a placebo-controlled, double-blind study design and use of a validated biomarker of erythrocyte membrane fatty acid content. Although it is clear that n-3 PUFA supplementation improves fatty acid status among AAA patients, further studies will be required to determine whether this improvement translates into positive alterations in the histopathologic appearance of AAA disease at the level of the aorta. In addition, while an aberrant fatty acid profile was observed in AAA patients compared to healthy control participants, it is not yet known why this occurs. It is possible that expression of fatty acid metabolising enzymes is dysregulated in AAA patients, resulting in the observed aberrant profile of enzyme activity and erythrocyte fatty acid proportions. It is noteworthy that participants were screened at entry for dietary intake of fish and seafood, with all participants consuming no more than two oily fish meals per week. It is therefore unlikely that the observed differences in fatty acid profile are attributed to differences in diet between the two cohorts.

Taken together, the results presented here indicate that erythrocytes from AAA patients demonstrate a distinct fatty acid profile that is characterised by aberrant proportions of n-6 fatty

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acids with high inflammatory potential while full blood count parameters among these patients are characterised by alterations that reflect the influence of systemic inflammation and oxidative stress. Improvements in inflammatory parameters and n-6 fatty acid status following n-3 PUFA supplementation suggest dietary fatty acids represent a viable therapeutic intervention in AAA.

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Table 1: Demographic, biometric and medical characteristics of AAA patients and control participants. Continuous demographic data are presented as mean $\pm$ SD, categorical demographic data are presented as number (percentage).

Variable	Observational Study		Omega-3 Clinical Trial	
	Control Participants (n=52)	AAA Patients (n=43)	AAA n-3 Cohort (n=15)	AAA Placebo Cohort (n=15)
Gender (Male/Female)	52/0	43/0	15/0	15/0
Age (years)	71.2 ± 5.9	74.6 ± 5.6 *	73.6 ± 5.0	75.1 ± 5.7
AAA size (mm)		38.8 ± 5.4	39.3 ± 5.7	39.2 ± 5.0
Smoking:				
Never	21 (40%)	9 (21%) †	1 (7%)	2 (13%)
Past	28 (54%)	30 (70%)	12 (80%)	12 (80%)
Current	3 (6%)	4 (9%)	2 (13%)	1 (7%)
BMI (kg/m^2)	25.9 ± 4.2	28.6 ± 4.8	29.4 ± 4.1	29.0 ± 5.0
SBP (mmHg)	137.3 ± 13.9	137.9 ± 16.2	135.8 ± 16.3	144.0 ± 19.6
DBP (mmHg)	79.3 ± 8.2	78.2 ± 8.8	77.6 ± 7.6	80.0 ± 10.7
Hypertension	20 (38%)	27 (63%) †	6 (40%)	11 (73%)
Diabetes	1 (2%)	6 (14%) †	3 (20%)	1 (7%)
Dyslipidaemia	19 (37%)	30 (70%) †	7 (47%)	12 (80%)
CHD	5 (10%)	18 (42%) †	5 (33%)	4 (27%)
<u>Medication</u>				
Anti-hypertensive agents:				
Beta blockers	7 (13%)	13 (30%)	4 (27%)	3 (20%)
AT II receptor antagonists	7 (13%)	13 (30%)	3 (20%)	5 (33%)
ACE inhibitors	7 (13%)	6 (14%)	3 (20%)	1 (7%)
Ca^{2+} channel blockers	5 (10%)	6 (14%)	1 (7%)	5 (33%)
Diuretics	3 (6%)	5 (12%)	2 (13%)	1 (7%)
Anti-platelet drugs	4 (8%)	25 (58%) †	11 (73%)	7 (47%)
NSAIDs	4 (8%)	6 (14%)	1 (7%)	2 (13%)
Statins	18 (35%)	32 (74%) †	8 (53%) ‡	13 (87%)

† AAA significantly different to control (Fisher's exact test, $p<0.05$)

* AAA significantly different to control (Student's t-test)

‡ AAA omega cohort significantly different to placebo cohort (Fisher's exact test, $p<0.05$)

BMI, body mass index; CHD, coronary heart disease; AT II, angiotensin II; Ca^{2+}, calcium; NSAIDs, non-steroidal anti-inflammatory drugs; SBP, systolic blood pressure; DBP, diastolic blood pressure

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Table 2: Erythrocyte fatty acid profiles of control participants and AAA patients (mean%±SEM)

Fatty Acid	Control	AAA
Saturated fatty acids		
C14:0	0.46±0.03	0.61±0.06
C15:0	0.17±0.01	0.26±0.04
C16:0	24.03±0.24	23.40±0.22
C17:0	0.28±0.01	0.34±0.01**
C18:0	14.23±0.13	14.23±0.13
C20:0	0.42±0.05	0.46±0.01
C22:0	3.16±0.14	3.17±0.14
Monounsaturated fatty acids		
C16:1n7	0.37±0.03	0.48±0.04
C18:1n-9	14.56±0.20	14.44±0.23
Trans fatty acids		
C18:1t	1.31±0.03	1.42±0.04
n-6 Polyunsaturated fatty acids		
C18:2	9.27±0.21	7.57±0.17**
C18:3	0.05±0.003	0.06±0.003
C20:2	0.15±0.01	0.16±0.01
C20:3	1.28±0.04	1.34±0.05
C20:4	12.52±0.18	12.89±0.22
C22:4	1.80±0.06	2.05±0.07
n-3 Polyunsaturated fatty acids		
C20:5	0.94±0.05	0.98±0.05
C22:5	1.84±0.03	1.96±0.05***
C22:6	3.13±0.10	3.46±0.11
Very long chain fatty acids		
C24:0	4.56±0.09	5.05±0.11
C24:1n9	4.70±0.10	5.27±0.1
C23:0	0.22±0.01	0.24±0.01
Other fatty acids		
C20:1n-7	0.18±0.01	0.16±0.01
Ratio n-6/n-3	4.08±0.13	3.61±0.11*
n-3 Index	4.07±0.13	4.42±0.12

* AAA significantly different to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)
Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age.

Table 3: Erythrocyte fatty acid ratios as indices of enzyme activities in control participants and AAA patients (Mean±SEM)

Index of enzyme activity	Control	AAA
Δ-4 desaturase (DHA/DPA)	1.72±0.06	1.81±0.07
Δ-9 desaturase (Oleic acid/stearic acid)	1.03±0.02	1.02±0.02
Δ-6 desaturase (1) Dihomo-γ-linolenic/linoleic	0.14±0.005	0.18±0.01**
Δ-desaturase (2) γ-Linolenic/linoleic	0.006±0.0004	0.008±0.0005
LA → AA biosynthesis (AA/LA)	1.37±0.06	1.75±0.06**
Stearoyl-CoA desaturase (Palmitoleic acid/palmitic acid)	0.015±0.001	0.02±0.002
Elongase 2/5 (Adrenic acid/AA)	0.14±0.005	0.16±0.03

* AAA significantly different to control (* $p<0.05$; ** $p<0.01$, *** $p<0.001$)
Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age

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Table 4: Red blood cell indices, white blood cell counts and platelet counts and indices for control participants and AAA patients (mean±SEM)

Blood Parameter	Control	AAA
WBC ($\times 10^9$/L)	6.1±0.2	6.7±0.3
Lymphocytes ($\times 10^9$/L)	1.9±0.1	2.1±0.1
Monocytes ($\times 10^9$/L)	0.4±0.02	0.4±0.1
Granulocytes ($\times 10^9$/L)	3.8±0.2	4.2±0.2
RBC ($\times 10^{12}$/L)	4.7±0.1	4.8±0.1
Hgb (g/L)	142.2±2.3	143.1±2.6
Hct (%)	43.5±0.6	43.7±0.7
MCV (fL)	91.8±0.8	92.1±0.7
MCH (pg)	30.0±0.3	30.1±0.3
MCHC (g/L)	326.9±1.5	327.2±1.8
RDW (%)	13.9±0.2	14.7±0.3
Platelet ($\times 10^9$/L)	240.6±10.8	199.9±10.8
MPV (fL)	7.8±0.2	8.0±0.2
Pct (%)	0.19±0.01	0.16±0.01
PDW	17.1±0.1	17.0±0.2

* AAA significantly different to control (* $p<0.05$); WBC, white blood cells; RBC, red blood cells; Hgb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width; MPV, mean plateletvolume; Pct, plateletcrit; PDW, platelet distribution width; Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age

Table 5: Erythrocyte fatty acid profiles of AAA patients (mean%±SEM).

Fatty Acid	AAA, Fish Oil Cohort			AAA, Placebo Cohort		
	Baseline	Week 3	Week 12	Baseline	Week 3	Week 12
Saturated fatty acids						
C14:0	0.71±0.12	0.71±0.06	0.51±0.06	0.73±0.09	0.67±0.08	0.58±0.06
C15:0	0.36±0.08	0.39±0.08	0.24±0.05	0.29±0.07	0.44±0.12	0.18±0.01
C16:0	22.71±0.22	23.65±0.32	23.49±0.36	22.63±0.30	22.73±0.23	23.22±0.43
C17:0	0.35±0.01	0.36±0.02	0.36±0.03	0.39±0.02	0.37±0.02	0.34±0.02
C18:0	14.15±0.25	14.39±0.25	14.98±0.43	14.44±0.23	14.08±0.22	14.62±0.26
C20:0	0.46±0.02	0.48±0.09	0.65±0.13	0.51±0.02	0.49±0.03	0.59±0.11
C22:0	3.27±0.28	2.69±0.29	2.98±0.28	3.04±0.28	3.02±0.31	3.20±0.25
Monounsaturated fatty acids						
C16:1n7	0.58±0.10	0.56±0.07	0.54±0.09	0.51±0.06	0.56±0.07	0.52±0.07
C18:1n-9	14.03±0.32	13.94±0.25	14.46±0.38	13.79±0.30	14.15±0.41	14.31±0.41
Trans fatty acids						
C18:1t	1.40±0.08	1.38±0.08	1.70±0.21	1.41±0.07	1.38±0.05	1.56±0.13
n-6 Polyunsaturated fatty acids						
C18:2	7.57±0.26	7.55±0.33	7.43±0.29	7.60±0.31	8.21±0.36	7.39±0.21
C18:3	0.06±0.005	0.09±0.04	0.05±0.01	0.06±0.005	0.06±0.004	0.05±0.01
C20:2	0.17±0.02	0.17±0.02	0.14±0.01	0.18±0.01	0.19±0.02	0.25±0.11
C20:3	1.32±0.05	1.14±0.05†	1.04±0.06†	1.51±0.08	1.52±0.09§§§	1.38±0.09§§
C20:4	12.46±0.23	11.72±0.22†	10.14±0.3†††	13.09±0.38	12.57±0.40	12.81±0.41§§§

C22:4	2.12±0.13	1.92±0.12	1.23±0.09†††	2.09±0.09	2.02±0.12	2.05±0.12§§§
n-3 Polyunsaturated fatty acids						
C20:5	0.99±0.07	1.35±0.06††	1.72±0.11†††	0.89±0.06	0.91±0.08§§§	0.95±0.09§§§
C22:5	1.97±0.09	1.87±0.08	1.61±0.08†	2.03±0.09	1.94±0.09	1.89±0.10
C22:6	3.54±0.22	4.72±0.19††	6.21±0.20†††	3.48±0.17	3.53±0.21§§§	3.31±0.24§§§
Very long chain fatty acids						
C24:0	5.25±0.19	5.04±0.17	4.92±0.24	5.44±0.12	5.34±0.16	5.23±0.20
C24:1n9	5.67±0.14	5.51±0.16	4.93±0.2†††	5.42±0.12	5.38±0.17	5.11±0.22
C23:0	0.24±0.02	0.26±0.01	0.24±0.02	0.28±0.01	0.29±0.01	0.25±0.02
Other fatty acids						
C20:1n-7	0.13±0.02	0.14±0.02	0.16±0.02	0.17±0.02	0.14±0.02	0.21±0.01§
Ratio n-6/n-3	3.48±0.16	2.69±0.08†††	2.00±0.08†††	3.66±0.20	3.68±0.22§§§	3.73±0.21§§§
n-3 Index	4.53±0.22	6.07±0.19†††	8.03±0.20†††	4.37±0.20	4.43±0.25§§§	4.26±0.28§§§

† Fish oil cohort week 3/week 12 significantly different to fish oil cohort baseline († $p<0.05$; †† $p<0.01$; ††† $p<0.001$)

§ Fish oil cohort week 3/week 12 significantly different to placebo cohort week 3/week 12 (§ $p<0.05$; §§ $p<0.01$; §§§ $p<0.001$)

Table 6: Erythrocyte fatty acid ratios as indices of enzyme activities in AAA patients (Mean±SEM)

Index of enzyme activity	Fish oil cohort		
	Baseline	Week 3	Week 12
Δ-4 desaturase (DHA/DPA)	1.85±0.14	2.60±0.16††	3.93±0.17†††
Δ-9 desaturase (Oleic acid/stearic acid)	1.00±0.03	0.97±0.03	0.98±0.04
Δ-6 desaturase (1) Dihomo-γ-linolenic/linoleic	0.18±0.01	0.15±0.01	0.15±0.01
Δ-desaturase (2) γ-Linolenic/linoleic	0.01±0.001	0.01±0.001	0.01±0.001
LA → AA biosynthesis (AA/LA)	1.68±0.08	1.60±0.09	1.40±0.07†
Stearoyl-CoA desaturase (Palmitoleic acid/palmitic acid)	0.03±0.004	0.02±0.003	0.02±0.003
Elongase 2/5 (Adrenic acid/AA)	0.17±0.01	0.16±0.01	0.12±0.01††

† Fish oil cohort week 3/week 12 significantly different to fish oil cohort baseline († $p<0.05$; †† $p<0.01$; ††† $p<0.001$)

Table 7: Red blood cell indices, white blood cell counts and platelet counts and indices for AAA patients (mean±SEM).

Blood Parameter	Fish Oil Cohort			Placebo Cohort		
	Baseline	Week 3	Week 12	Baseline	Week 3	Week 12
WBC (x10^9/L)	6.9±0.5	6.3±0.5	6.6±0.4	6.6±0.5	6.5±0.4	6.7±0.5
Lymphocytes (x10^9/L)	2.1±0.1	2.0±0.2	2.1±0.2	2.1±0.2	1.8±0.2	1.9±0.2
Monocytes (x10^9/L)	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.05	0.3±0.02	0.5±0.1
Granulocytes (x10^9/L)	4.4±0.4	3.8±0.3	4.0±0.3	4.1±0.3	4.3±0.3	4.4±0.4
RBC (x10^{12}/L)	4.7±0.1	4.6±0.1	4.6±0.1†	4.8±0.1	4.7±0.1	4.8±0.2
Hgb (g/L)	144.1±3.5	142.5±4.0	139.2±3.6	143.8±3.9	142.2±4.5	143.7±5.1
Hct (%)	44.1±1.0	43.5±1.1	42.5±1.1†	43.7±1.1	43.5±1.3	43.8±1.5
MCV (fL)	93.5±0.9	94.6±1.2	93.6±1.2	91.0±0.9	91.8±1.0	91.9±1.2
MCH (pg)	30.5±0.3	31.0±0.5	30.7±0.5	30.0±0.4	30.0±0.4	30.2±0.6
MCHC (g/L)	326.6±2.8	327.1±3.2	328.3±2.2	328.8±2.4	326.6±1.9	328.0±2.3
RDW (%)	14.8±0.4	14.5±0.4	13.8±0.2††	14.4±0.3	14.7±0.3	14.0±0.2
Platelet (x10^9/L)	207.9±16.0	205.5±21.1	198.3±14.1†	192.8±16.3	194.4±19.2	191.3±24.1
MPV (fL)	8.0±0.3	7.5±0.4	7.7±0.2	7.7±0.2	7.8±0.2	8.0±0.2
Pct (%)	0.17±0.01	0.15±0.02	0.15±0.01†	0.15±0.01	0.15±0.02	0.15±0.02
PDW	16.8±0.2	17.0±0.2	16.9±0.2	17.3±0.2	17.0±0.3	17.3±0.3

† Fish oil cohort week 3/week 12 significantly different to fish oil cohort baseline († $p<0.05$; †† $p<0.01$); WBC, white blood cells; RBC, red blood cells; Hgb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; Pct, plateletcrit; PDW, platelet distribution width

Table 8: Independent associations between AAA diagnosis and assessed variables using multi-variable regression analysis

Variable	β	Standard Error	95% CI	P value
Margaric acid	0.509	0.029	0.025-0.144	0.007
Linoleic acid	-0.522	0.541	-2.640-(-0.446)	0.007
Docosapentaenoic acid	0.715	0.115	0.260-0.725	<0.001
Δ6-Desaturase (1)	0.538	0.018	0.017-0.089	0.005
LA →AA biosynthesis	0.516	0.143	0.110-0.689	0.008
n-6/n-3 Ratio	-0.403	0.334	-1.1364-(-0.010)	0.047

Data was adjusted for hypertension, diabetes mellitus, coronary heart disease, low-dose aspirin and statin use, active or previous smoking history and age. 95% CI, 95% Confidence interval. No independent associations were observed for red blood cell distribution width, omega-3 index, adrenic acid, arachidonic acid (AA), myristic acid, pentadecanoic acid, palmitoleic acid, elaidic acid, γ-linolenic acid, DHA, lignoceric acid, nervonic acid, tricosanoic acid, stearoyl-CoA desaturase, elonase-2/5, or Δ6-Desaturase (2). LA, linoleic acid; AA arachidonic acid
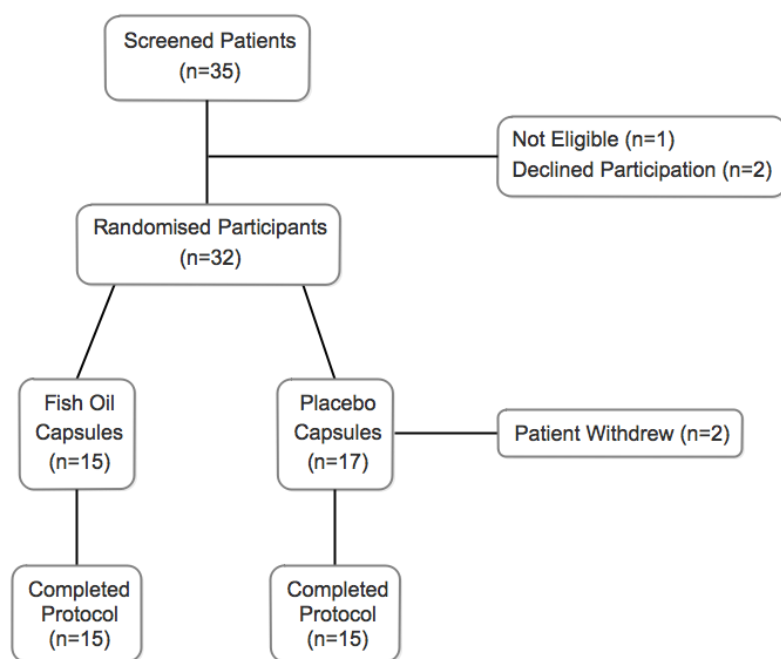

Figure 1: Study flowchart. The intervention study included 32 AAA patients, 30 of which completed the protocol.