Journal of Nutrition & Intermediary Metabolism 5 (2016) 61–69

Contents lists available at ScienceDirect

Journal of Nutrition & Intermediary Metabolism

journal homepage: http://www.jnimonline.com/

# Erythrocyte omega-3 polyunsaturated fatty acid levels are associated with biomarkers of inflammation in older Australians

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ARTICLE INFO

*Article history:*
Received 30 November 2015
Received in revised form
16 March 2016
Accepted 18 March 2016
Available online 30 March 2016

*Keywords:*
Omega-3 polyunsaturated fatty acids
Omega-3 index
Inflammation
C-reactive protein
Fibrinogen
White blood cells
Monocytes
Neutrophils
Ageing

ABSTRACT

*Background:* Elevated levels of pro-inflammatory mediators heighten the risk of developing or aggravating a spectrum of chronic diseases and are a strong predictor of mortality in elderly cohorts. Omega-3 polyunsaturated fatty acids (n-3PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to possess anti-inflammatory properties. However, the relationship between erythrocyte membrane n-3PUFA and inflammation biomarkers has not been well established.

*Objective:* This study aimed to determine if n-3PUFA status, together with the omega-3 index (O3I, erythrocyte membrane % EPA plus DHA), is associated with pro-inflammatory mediators in older Australians.

*Methods:* The study was a cross-sectional analysis of randomly selected older men and women aged ≥65 years (n = 620) recruited from the Central Coast of NSW, Australia. Fasted blood samples were analysed for C-reactive protein (CRP), fibrinogen and full blood count using standardised laboratory methods. The fatty acid composition of erythrocyte membranes was analysed via gas chromatography to determine n-3PUFA levels. The relationships between n-3PUFA and inflammatory mediators were evaluated in multivariate regression models after adjusting for known inflammatory confounders.

*Results:* After excluding participants who had an inflammatory disease, CRP levels >10 mg/L, or who were taking anti-inflammatory medications or n-3PUFA supplements, 126 participants (age 77.6 ± 7.3 years; females, 46%) were included in the analysis. After multivariate adjustments, O3I was inversely associated with CRP ($\beta = -0.209$, $p < 0.05$) and monocyte cell counts ($\beta = -0.205$, $p < 0.05$), and total n-3PUFA was inversely related to WBC ($\beta = -0.238$, $p < 0.05$), neutrophils ($\beta = -0.212$, $p < 0.05$) and monocytes ($\beta = -0.246$, $p < 0.05$). However no association between fibrinogen and O3I or total n-3PUFA was detected.

*Conclusions:* This study demonstrated a negative association between O3I and biomarkers of inflammation in an older population. The findings support a potential role for n-3PUFA supplementation in the management of inflammatory diseases.



## 1. Introduction

Pro-inflammatory mediators (including cytokines and eicosanoids) have been shown to be elevated in elderly people by as much as two-to four-fold, when compared to a younger population [1]. Sustained low grade inflammation indicated by increased circulating levels of C-reactive protein (CRP) is a strong predictor of mortality in elderly cohorts irrespective of concurrent morbidities or other known risk factors such as smoking or high blood pressure [1]. The inflammatory process is designed to help fight infection, remove harmful substances and repair damaged tissue and organ systems [2]. Although this process is generally protective, failure to resolve the inflammatory response can result in chronic inflammation, which may lead to the development and progression of a

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http://dx.doi.org/10.1016/j.jnim.2016.03.002

spectrum of chronic diseases including coronary vascular disease, Alzheimer's disease, depression, rheumatoid arthritis, cancer, sarcopenia and reduced physical functioning [1,3–5].

Elevated levels of acute phase proteins, C-reactive protein (CRP) and fibrinogen are reliable markers of sustained low grade systemic inflammation [6–8]. CRP and fibrinogen are released by hepatocytes in response to elevated cytokine levels during the acute inflammatory response. Fibrinogen is also a principal protein involved in blood clotting [7].

Omega-3 polyunsaturated fatty acids (n-3PUFA) of marine origin have attracted attention due to their anti-inflammatory effects [9]. The proposed mechanisms by which n-3PUFA mediates these effects include preferential incorporation of n-3PUFA into phospholipids resulting in the production of 3-series eicosanoids with lower biological activity, production of inflammation resolving lipid mediators (resolvins and protectins) and altered cytokine gene expression [2].

Most epidemiological and experimental studies have shown an association between higher plasma n-3PUFA levels and reductions in circulating plasma inflammatory mediators; interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-$\alpha$) and CRP [10–13]. However, randomised controlled trials involving n-3PUFA supplementation have produced inconsistent results. Rangel-Heurta et al. were unable to draw a clear conclusion on the association between plasma n-3PUFA levels and reductions in inflammatory mediators, due to heterogeneity in study methodologies that include variations in study population, supplement dosage, study length, and sample size [14]. The strongest associations between plasma n-3PUFA levels and inflammatory mediators have been found in older populations, and in populations with elevated inflammatory biomarkers [15,16]. More recently a meta-analysis of randomised controlled trials by Li et al. reported a reduction in inflammatory mediators upon n-3PUFA supplementation in healthy and diseased populations [17].

The omega-3 index (O3I) is defined as the sum of EPA and DHA expressed as a percentage of total erythrocyte fatty acids [18]. O3I has been shown to correlate with n-3PUFA levels in body tissues [18] and is considered a reliable biomarker of long term n-3PUFA intake. The determination of O3I provides an opportunity to examine potential associations between n-3PUFA dietary intake and reductions in inflammatory biomarkers such as CRP and fibrinogen. To date, the association of O3I levels and inflammatory biomarkers has only been explored within unhealthy or moderately hypertriglyceridemic populations and has produced inconsistent results [12,19,20]. The aim of this project was to determine if n-3PUFA status, and in particular O3I, is associated with inflammatory mediators in older Australians. Our recent studies have demonstrated that the association between n-3PUFA status and chronic disease risk factors is sex-dependent [21,22], therefore, we examined the relationship between n-3PUFA and inflammation mediators separately in older men and women.

## 2. Materials and methods

### 2.1. Study population and design

This study is a cross-sectional analysis of data collected as part of the Retirement Health and Lifestyle Study (RHLS); a study of the health and lifestyle of older Australians (65 years and older) residing in retirement villages or within the community in the Central Coast region of NSW, Australia. Participants were eligible for the RHLS if they: were $\geq$65 years of age; their primary residence was located within the Wyong or Gosford Local Government Areas; and they had been living at their current address for $\geq$12 months. Participants were not eligible if: they were not living independently or were residing in a communal setting other than a retirement village; another member of their household was taking part in the study; they had language and/or other communicative difficulties that limited participation; or they were cognitively impaired and/or were unable to provide informed consent. Those who were eligible and chose to participate in the study (n = 831) took part in an interviewer-administered questionnaire (IAQ) adapted from validated lifestyle surveys [21–24]. A subset of eligible participants completed a clinical assessment (n = 670) and provided a blood sample (n = 649). Participants were included in the present study if they had erythrocyte samples available for fatty acid analyses (n = 620) and valid anthropometric and inflammatory marker measurements. Participants with CRP levels $\geq$10 mg/L (indicative of acute inflammation), and/or who reported inflammatory disease (e.g. arthritis), and/or that they were taking anti-inflammatory medication or n-3PUFA supplements [25] were excluded from the analyses. All subjects provided written informed consent, and the study was approved by the University of Newcastle Human Research Ethics Committee (H-2008-0431) and the Northern Sydney Central Coast Health Human Research Ethics Committee (Reference No. 1001-031M). The participant recruitment process is summarised in Fig. 1.

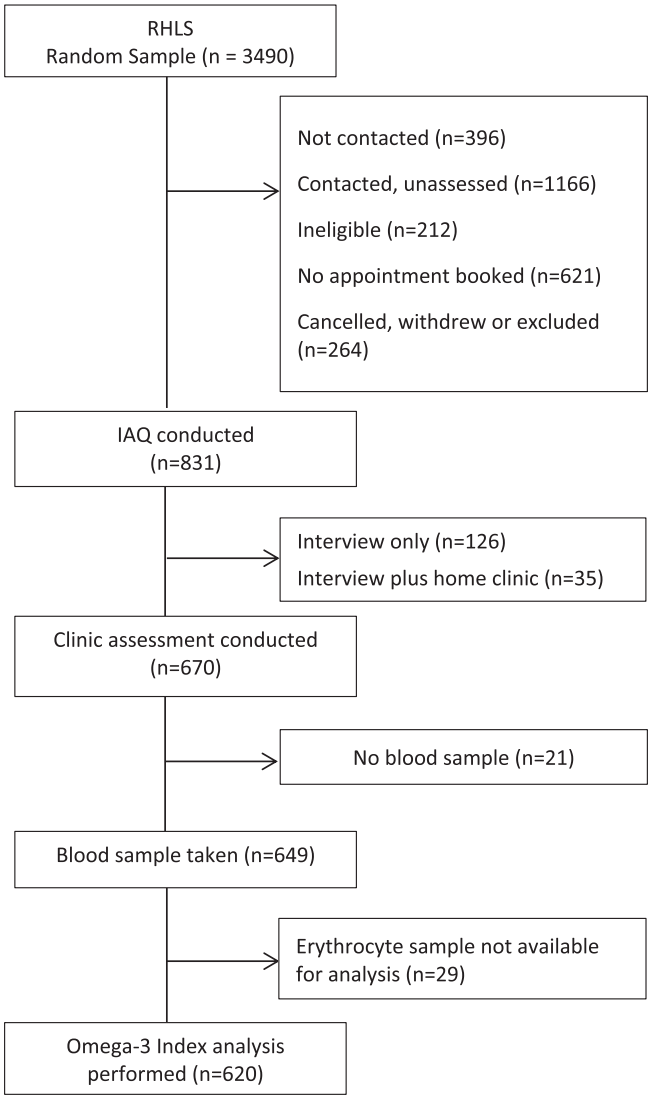


**Fig. 1.** Overview of participant recruitment.

### 2.2. Biochemical analyses

Blood samples were collected from subjects in a fasting state (>10 h) by a trained phlebotomist. Participants were asked to abstain from smoking on the morning of collection and to rest for 5 min prior to sample collection. CRP, fibrinogen, and white blood cell counts (WBC) including WBC differentials were analysed using standardised methodology by an accredited pathology service (Hunter New England Area Pathology Service).

For erythrocyte membrane fatty acid composition determination, blood samples were first centrifuged at 3000*g* for 10 min to separate the plasma and erythrocyte fractions. Fatty acid profiles were analysed using the method of Lepage and Roy via direct transesterification of the washed red blood cell (RBC) fraction followed by gas chromatography [26]. Methanol:toluene (2 mL, 4:1 v/v, containing C19:0 as internal standard) was added to the erythrocyte samples. Acetyl chloride (200 μL) was added while vortexing and the samples were then heated for 1 h at 100 °C. After the samples were cooled in water for 5 min, $K_2CO_3$ 6% (5 mL) was added and the samples were centrifuged (3000*g*, 5 min, 4 °C). The upper toluene phase was collected and stored at −20 °C until GC analysis.

Methylated fatty acid samples were analysed using a Hewlett Packard 6890 Series GC equipped with a fixed carbon-silica column 30 m × 0.25 mm (DB-225) (J and W Scientific), flame ionization detector, autosampler and autodetector. Injector and detector ports were set at 250 °C. The oven temperature was programmed at: 170 °C for two minutes then increased 10 °C per minute to 190 °C where it remained stationary for one minute, then increased 3 °C per minute to 220 °C, and held for a total run time of 30 min per sample. A split ratio of 10:1 and an injection volume of 3 μL were used. Sample peaks were identified by comparison of retention times to known fatty acid standards. Fatty acid profile data were stored and processed using Hewlett Packard Chemstations Version A. 04.02 software. The O3I was calculated by summing the peak area of erythrocyte membrane EPA and DHA and was expressed as a percentage of the total peak area for all erythrocyte membrane fatty acids measured and total n-3PUFA was calculated by summing α-linolenic acid (ALA), EPA, docosapentaenoic acid (DPA) and DHA.

### 2.3. Demographic, physical activity, medical history and dietary intake

Demographic data (sex, age, marital status, income, employment, education and country of birth), and information about smoking and participation in physical activity, was collected via an IAQ. Information about diagnosed conditions, medical history and health status was collected via a self-administered medical history questionnaire. Food intake was assessed via a self-administered food frequency questionnaire (FFQ) adapted from the validated interviewer administered CSIRO FFQ [27]. Dietary data was analysed using FoodWorks Professional Edition 2009, Version 6.0.2562 (Xyris software, Brisbane, QLD, Australia).

### 2.4. Anthropometrics

Anthropometric measures, including height, weight, waist and hip circumference, were taken by trained research officers according to World Health Organisation guidelines [28]. Body weight in light clothing and without shoes was measured using a digital scale (Tanita HD316, Tanita Corporation, Tokyo, Japan; or Wedderburn UWPM150; Wedderburn Scales, Australia) to the nearest 100 g, with subject's weight evenly distributed on the scales. Height was measured using a portable stadiometer (Design No.1013522, Surgical & Medical Products, Australia) to the nearest 0.1 cm. Waist circumference was measured at the mid-point of the waist between the lower coastal arch and the iliac crest using a soft measuring tape, to the nearest 0.1 cm and upon normal expiration. Hip circumference was measured at the greatest posterior protuberance of the gluteal to the nearest 0.1 cm. Measurement was taken upon normal expiration. All anthropometric measurements were taken twice. If the measurements disagreed by more than set tolerance limits a third measure was taken. Each measure was presented as the mean of the two observations or the mean of the two closest measurements if a third was taken. The mean height and weight were used to calculate body mass index (kg/m$^2$).

### 2.5. Sitting blood pressure

Seated blood pressure was measured by trained staff using a calibrated Omron 1A2 digital automatic blood pressure monitor (Omron, Australia) as per the National Heart Foundation Guidelines [29]. Participants were asked to avoid strenuous exercise for 24 h prior to measurement, fast overnight, and abstain from smoking on the morning of measurement. Serial readings were taken (minimum of three), and the average of the last two readings within 10 mmHg SBP and 6 mmHg DBP were recorded.

### 2.6. Statistical analysis

Data was analysed using Statistical Package for the Social Sciences (SPSS; Release 17.0, Chicago, IL: SPSS Inc). Descriptive statistics were undertaken for total group, men and women. Continuous data was reported as mean ± standard deviation (SD). Categorical data were reported as frequencies and/or percentages. Group differences between male and female subgroups were assessed using independent samples t-tests (two-tailed) and chi-square analyses. Pearson product-moment correlation analyses were used to evaluate the association between erythrocyte membrane n-3PUFA levels and plasma inflammatory mediators (CRP, fibrinogen, WBCs and WBC differentials). Differences in the number of participants with pro-inflammatory mediator levels above and within reference ranges in O3I quartiles was evaluated using a linear-by-linear association chi-square test [30,31].

Two-block hierarchical multiple regression analyses were used to examine the relationship between O3I and total n-3PUFA and specific inflammatory mediators [32,33]. Anthropometric, demographic and other potentially confounding variables (age, sex, BMI, systolic blood pressure, smoking status, sedentary lifestyle, fasting blood glucose, total cholesterol and triglycerides) were entered into the regression analyses at block 1. Either O3I or total n-3PUFA was entered at the second block of the analysis. The multivariate relationships between select inflammatory mediators and O3I or total n-3PUFA were analysed separately to avoid co-linearity. Pairwise exclusion for missing data was employed in all analyses. Statistical significance was set at $p < 0.05$.

## 3. Results

After excluding those participants who reported a pre-existing inflammatory disease and/or had CRP levels >10 mg/L (n = 236), together with those who were taking anti-inflammatory medications (n = 221) or n-3PUFA supplements (n = 37), 126 participants (age 77.6 ± 7.3 years; females 46%) remained in the analysis (Table 1). As expected there was a significant difference between males and females for height ($p < 0.001$), body weight ($p < 0.001$), and waist circumference ($p < 0.001$), being higher in men. Waist-to-hip ratio (WHR) was also significantly higher in men ($p < 0.001$). The mean waist circumference for men and women were both above the National Heart Foundation defined cut-off points (men, <94 cm; women, <80 cm) for increased risk of heart

**Table 1**
Characteristics of the study participants.[a]

| | All (n = 126) | Men (n = 68) | Women (n = 58) | *p* |
|---|---|---|---|---|
| Age (years) | 77.6 ± 7.3 | 78.3 ± 7.2 | 76.9 ± 7.3 | NS |
| Current smoker (n, %) | 3 (2.4%) | 2 (2.9%) | 1 (1.7%) | NS |
| Physical inactivity (n, %) | 8 (6.3%) | 4 (5.9%) | 4 (6.9%) | NS |
| Height (m) | 1.65 ± 0.09 | 1.70 ± 0.07 | 1.59 ± 0.07 | <0.001 |
| Weight (kg) | 74.4 ± 14.6 | 79.8 ± 14.1 | 68.3 ± 12.6 | <0.001 |
| BMI (kg/m$^2$) | 27.0 ± 4.0 | 27.2 ± 3.9 | 26.9 ± 4.2 | NS |
| Waist (cm) | 96.8 ± 12.1 | 101.8 ± 10.9 | 90.9 ± 10.9 | <0.001 |
| Hip (cm) | 106.8 ± 8.6 | 106.3 ± 6.9 | 107.3 ± 10.3 | NS |
| Waist: Hip ratio | 0.91 ± 0.08 | 0.95 ± 0.06 | 0.85 ± 0.05 | <0.001 |
| SBP (mmHg) | 147.3 ± 21.2 | 150.3 ± 19.4 | 143.9 ± 22.8 | NS |
| DBP (mmHg) | 75.1 ± 10.1 | 76.4 ± 9.7 | 73.5 ± 10.4 | NS |
| Total protein (g/L) | 76.3 ± 4.3 | 76.7 ± 4.1 | 75.9 ± 4.6 | NS |
| Glucose (mmol/L) | 5.5 ± 1.0 | 5.6 ± 1.0 | 5.4 ± 0.9 | NS |
| Total cholesterol (mmol/L) | 4.7 ± 1.0 | 4.5 ± 0.9 | 5.0 ± 1.0 | <0.01 |
| Triglycerides (mmol/L) | 1.2 ± 0.5 | 1.2 ± 0.5 | 1.3 ± 0.6 | NS |
| LDL (mmol/L) | 2.6 ± 0.9 | 2.5 ± 0.8 | 2.8 ± 0.9 | <0.05 |
| HDL (mmol/L) | 1.5 ± 0.5 | 1.4 ± 0.4 | 1.7 ± 0.5 | <0.01 |

BMI = body mass index, DBP = diastolic blood pressure, SBP = systolic blood pressure, HDL = high-density lipoproteins, LDL = low-density lipoproteins, NS = not statistically significant at $p < 0.05$.
[a] Data are presented as mean and standard deviation or number and (%). Continuous variables were compared across gender subgroups using two-tailed independent samples student t-tests. Categorical variables were compared across gender subgroups using Pearson's chi-square tests.

disease [34]. Mean total cholesterol ($p < 0.01$), LDL-cholesterol ($p < 0.05$) and HDL-cholesterol ($p < 0.01$) was higher in women, while mean systolic and diastolic blood pressure was similar between males and females. The prevalence of smoking and physical inactivity was also similar across the two genders (Table 1). There were no significant differences in the dietary intake of macronutrients between male and female participants (Table 2). Compared to males, female participants had higher erythrocyte membrane DHA ($p < 0.05$), O3I ($p < 0.05$), and total n-3PUFA ($p < 0.05$) (Table 3).

Females had significantly lower monocyte ($p < 0.001$) and eosinophil ($p < 0.05$) cell counts than males, while total WBC, neutrophil, lymphocyte and eosinophil cell counts, and plasma fibrinogen and CRP levels were not significantly different between the gender sub-groups (Table 4).

The results of bivariate correlation analyses of the relationships between erythrocyte membrane n-3PUFA and the various inflammatory markers are presented in Table 5. With the exception of the inverse relationship detected between fibrinogen and DPA in male participants, fibrinogen, lymphocytes and basophils were not correlated with any of the n-3PUFA reviewed. In contrast, CRP, WBC, neutrophils, monocytes and eosinophils displayed negative correlations with a number of n-3PUFA. CRP was inversely correlated to DHA, O3I and total n-3PUFA. These relationships were not gender specific, with similar but non-significant trends in association evident in both gender subgroups. WBC and monocytes were negatively correlated with EPA, DPA, DHA, and consequently O3I and total n-3PUFA in the analysis of the overall group. Neutrophils were also negatively correlated with all n-3PUFA variables with the exception of DHA. Gender specificity was evident, with the relationships between WBC and n-3PUFA and neutrophils and n-3PUFA generally displaying specificity to males, and the relationship between monocytes and DPA displaying specificity to females. Eosinophils were also inversely correlated to n-3PUFA in women, but not men, with significant correlations detected between eosinophils and EPA, DHA, O3I and total n-3PUFA.

Hierarchical regression analyses were conducted to assess the association between key inflammatory markers (CRP, WBC, neutrophils, monocytes, eosinophils) and n-3PUFA variables (O3I, total n-3PUFA) after adjusting for potentially confounding variables (age, gender, BMI, systolic blood pressure, sedentary lifestyle, fasting glucose, total cholesterol and triglycerides). The multivariate relationships between each inflammatory marker and O3I, or total n-3PUFA, were assessed separately and the results are summarised in Table 6.

O3I, and total n-3PUFA levels, were significantly associated with monocyte cell counts in all participants, after correcting for the potentially confounding influences of age, gender, BMI, systolic blood pressure, sedentary lifestyle, fasting glucose, total cholesterol and triglycerides (O3I: $R = 0.433$, $p < 0.05$; total n-3PUFA: $R = 0.449$, $p < 0.01$). In both models, the addition of the n-3PUFA variable at the second block of the analyses resulted in significant

**Table 2**
Nutrient intake of the study participants.[a]

| | All (n = 126) | Men (n = 68) | Women (n = 58) | *p* |
|---|---|---|---|---|
| Total energy (kJ/d) | 7648±2862 | 7984±2930 | 7254±2755 | NS |
| Total lipid (g/d) | 66.8±29.5 | 67.4±29.4 | 66.1±29.8 | NS |
| MUFA (g/d) | 25.6±11.8 | 25.7±12.1 | 25.4±11.6 | NS |
| PUFA (g/d) | 10.9±7.4 | 11.1±7.1 | 10.7±7.8 | NS |
| SFA (g/d) | 23.7±11.8 | 23.9±11.9 | 23.5±11.8 | NS |
| Carbohydrate (g/d) | 193.6±80.4 | 204.2±83.8 | 181.2±75.0 | NS |
| Protein (g/d) | 84.8±34.3 | 87.1±30.9 | 82.1±37.9 | NS |

MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids, NS = not statistically significant at $p < 0.05$.
[a] Data are presented as mean and standard deviation. Gender subgroups were compared using two-tailed independent samples student t-tests.

**Table 3**
Erythrocyte fatty acids (%) of study participants.[a]

| | All (n = 126) | Men (n = 68) | Women (n = 58) | p |
|---|---|---|---|---|
| **SFA** | | | | |
| 16:0 | 22.77±1.44 | 22.81±1.66 | 22.72±1.16 | NS |
| 18:0 | 18.48±1.44 | 18.46±1.64 | 18.51±1.17 | NS |
| 20:0 | 0.64±0.23 | 0.62±0.24 | 0.66±0.21 | NS |
| Total SFA | 41.89±2.44 | 41.88±2.80 | 41.89±1.96 | NS |
| **MUFA** | | | | |
| 16:1n-7 | 0.55±0.28 | 0.54±0.32 | 0.56±0.24 | NS |
| 18:1n-7 | 1.75±0.41 | 1.73±0.44 | 1.76±0.38 | NS |
| Total MUFA | 2.30±0.60 | 2.28±0.67 | 2.32±0.52 | NS |
| **n-6PUFA** | | | | |
| 18:2n-6 | 9.11±1.62 | 9.15±1.79 | 9.06±1.40 | NS |
| 18:3n-6 | 0.22±0.30 | 0.23±0.37 | 0.22±0.20 | NS |
| 20:2n-6 | 0.34±0.32 | 0.37±0.42 | 0.30±0.11 | NS |
| 20:3n-6 | 1.46±1.11 | 1.45±1.12 | 1.47±1.11 | NS |
| 20:4n-6 | 17.04±2.33 | 17.14±2.30 | 16.92±2.38 | NS |
| Total n-6PUFA | 28.16±2.44 | 28.34±2.62 | 27.96±2.23 | NS |
| **n-3PUFA** | | | | |
| 18:3n-3 (ALA) | 0.28±0.15 | 0.28±0.16 | 0.28±0.15 | NS |
| 20:5n-3 (EPA) | 1.38±0.65 | 1.28±0.62 | 1.49±0.67 | NS |
| 22:5n-3 (DPA) | 3.46±0.63 | 3.49±0.65 | 3.42±0.62 | NS |
| 22:6n-3 (DHA) | 6.28±1.44 | 5.98±1.40 | 6.64±1.41 | <0.05 |
| Total n-3PUFA | 11.40±2.14 | 11.03±2.11 | 11.83±2.11 | <0.05 |
| Omega-3 Index | 7.66±1.92 | 7.27±1.83 | 8.13±1.94 | <0.05 |

ALA = alpha-linolenic acid, DHA = docosahexaenoic acid, DPA = docosapentaenoic acid, EPA = eicosapentaenoic acid, MUFA = monounsaturated fatty acids, n-3PUFA = omega-3 polyunsaturated fatty acids, Omega-3 index = Erythrocyte EPA + DHA % total fatty acids, n-6PUFA = omega-6 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids, NS = not statistically significant at $p < 0.05$.

[a] Data are presented as mean and standard deviation. Gender subgroups were compared using two-tailed independent samples student t-tests.

**Table 4**
Biomarkers of inflammation in study participants.[a]

| | All (n = 126) | Men (n = 68) | Women (n = 58) | p |
|---|---|---|---|---|
| **Markers of Inflammation** | | | | |
| Fibrinogen (g/L) | 3.5±0.6 | 3.4±0.5 | 3.6±0.7 | NS |
| CRP (mg/L) | 2.4±2.0 | 2.3±1.7 | 2.6±2.3 | NS |
| **WBC & WBC differentials** | | | | |
| WBC ($10^9$/L) | 6.29±1.64 | 6.55±1.64 | 6.00±1.61 | NS |
| Neutrophils ($10^9$/L) | 3.81±1.28 | 3.98±1.20 | 3.61±1.34 | NS |
| Lymphocyte ($10^9$/L) | 1.71±0.54 | 1.70±0.53 | 1.73±0.55 | NS |
| Monocytes ($10^9$/L) | 0.54±0.21 | 0.61±0.23 | 0.46±0.15 | <0.001 |
| Eosinophils ($10^9$/L) | 0.20±0.15 | 0.23±0.18 | 0.16±0.09 | <0.05 |
| Basophils ($10^9$/L) | 0.02±0.04 | 0.02±0.04 | 0.02±0.04 | NS |

CRP = C-reactive protein, WBC = white blood cell, NS = not statistically significant at $p < 0.05$.

[a] Data are presented as mean and standard deviation. Gender subgroups were compared using two-tailed independent samples student t-tests.

increases in prediction (O3I: $\Delta R^2 = 3.5\%$, $p < 0.05$; total n-3PUFA: $\Delta R^2 = 4.9\%$, $p < 0.05$). O3I and total n-3PUFA were also independent negative predictors of monocyte counts in their respective models (O3I: $\beta = -0.205$, $p < 0.05$; total n-3PUFA: $\beta = -0.246$, $p < 0.05$); as was gender (O3I: $\beta = -0.300$, $p < 0.01$; total n-3PUFA: $\beta = -0.309$, $p < 0.01$), with female sex being associated with lower monocyte counts.

Hierarchical regression analyses of WBC counts against total n-3PUFA also resulted in a significant predictive model and multivariate R value, after adjustment for potentially confounding variables ($R = 0.397$, $p < 0.05$). In this analysis, the addition of total n-3PUFA significantly increased the model's predictive power ($\Delta R^2$: 4.6%, $p < 0.05$) and the negative correlation between total n-3PUFA and WBC counts was the only relationship that contributed

**Table 5**
Correlations between erythrocyte fatty acids and biomarkers of inflammation.[a]

| Biomarker | 20:5n-3 EPA | 22:5n-3 DPA | 22:6n-3 DHA | Omega-3 Index | Total n-3PUFA |
|---|---|---|---|---|---|
| **Fibrinogen** | | | | | |
| All | −0.139 | −0.138 | −0.050 | −0.084 | −0.112 |
| Men | −0.150 | −0.310* | −0.057 | −0.095 | −0.178 |
| Women | −0.175 | 0.010 | −0.100 | −0.133 | −0.110 |
| **CRP** | | | | | |
| All | −0.160 | −0.062 | −0.182* | −0.190* | −0.193* |
| Men | −0.187 | −0.170 | −0.148 | −0.177 | −0.211 |
| Women | −0.172 | 0.042 | −0.258 | −0.246 | −0.218 |
| **WBC** | | | | | |
| All | −0.213* | −0.255** | −0.177* | −0.204* | −0.257** |
| Men | −0.278* | −0.306* | −0.153 | −0.212 | −0.286* |
| Women | −0.095 | −0.221 | −0.133 | −0.130 | −0.169 |
| **Neutrophils** | | | | | |
| All | −0.227* | −0.227* | −0.172 | −0.206* | −0.247** |
| Men | −0.272* | −0.351** | −0.100 | −0.169 | −0.261* |
| Women | −0.146 | −0.114 | −0.190 | −0.188 | −0.188 |
| **Lymphocytes** | | | | | |
| All | 0.008 | −0.148 | 0.007 | 0.008 | −0.044 |
| Men | −0.157 | −0.056 | −0.156 | −0.173 | −0.175 |
| Women | 0.174 | −0.256 | 0.180 | 0.191 | 0.093 |
| **Monocytes** | | | | | |
| All | −0.227* | −0.179* | −0.243** | −0.258** | −0.283** |
| Men | −0.179 | −0.151 | −0.166 | −0.188 | −0.213 |
| Women | −0.208 | −0.330* | −0.214 | −0.227 | −0.293* |
| **Eosinophils** | | | | | |
| All | −0.124 | −0.015 | −0.178* | −0.175 | −0.162 |
| Men | −0.014 | −0.015 | −0.047 | −0.041 | −0.048 |
| Women | −0.271* | −0.060 | −0.354** | −0.351** | −0.323* |
| **Basophils** | | | | | |
| All | −0.075 | −0.044 | −0.029 | −0.047 | −0.040 |
| Men | −0.170 | 0.022 | 0.032 | 0.019 | 0.032 |
| Women | −0.130 | −0.135 | −0.086 | −0.107 | −0.114 |

ALA = alpha-linolenic acid, CRP = C-reactive protein, DHA = docosahexaenoic acid, DPA = docosapentaenoic acid, EPA = eicosapentaenoic acid, MUFA = monounsaturated fatty acids, n-3PUFA = omega-3 polyunsaturated fatty acids, Omega-3 index = Erythrocyte EPA + DHA % total fatty acids, n-6PUFA = omega-6 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, SFA = saturated fatty acids, WBC = white blood cell.

[a] Pearson bivariate two-tailed correlation analyses were used to test associations. Pearson's correlation coefficient (r) presented.*<0.05** < 0.01.

independently and significantly to the multivariate association observed. A similar but non-significant trend in association was evident for the adjusted analyses of the relationship between WBC and O3I.

Separate hierarchical regression analyses of O3I and total n-3PUFA against CRP, neutrophils and eosinophils, adjusted for age, gender, BMI, systolic blood pressure, sedentary lifestyle, fasting glucose, total cholesterol and triglycerides, resulted in non-significant models with multivariate correlation coefficients between 0.311 and 0.349. Despite the lack of model significance, the addition of O3I or total n-3PUFA at the second block of the analyses contributed significantly to the prediction of CRP and neutrophil cell counts, respectively ($\Delta R^2$ for both models = 3.6%, $p < 0.05$). O3I and total n-3PUFA were also the only independent variables to exert a significant independent influence on CRP levels ($\beta = -0.215$, $p = 0.04$) and neutrophil cell counts ($\beta = -0.215$, $p = 0.04$), with higher levels of n-3PUFA associated with lower levels of the inflammatory markers.

The percentage of participants with CRP levels within the reference range for each O3I quartile is presented in Fig. 2. The proportion of participants with low to average CRP levels (≤3.0 mg/L) increased across increasing O3I quartiles (61.3%, 74.2%, 68.8%, 84.4%, $p$-trend = 0.04), with significant differences in the proportion of participants with CRP levels ≤3 mg/L found between the first (61.3%) and fourth quartiles (84.4%; $\chi^2 = 4.26$, $p = 0.04$).

**Table 6**
Hierarchical regression analysis of the relationship between inflammatory markers and (a) O3I and (b) total n-3PUFA.[a]

| | CRP | | WBC | | Neutrophils | | Monocytes | | Eosinophils | |
|---|---|---|---|---|---|---|---|---|---|---|
| **(a) & (b) Model 1** | $R = 0.257$, $R^2 = 0.066$, p = ns | | $R = 0.334$, $R^2 = 0.112$, p = ns | | $R = 0.292$, $R^2 = 0.085$, p = ns | | $R = 0.390$, $R^2 = 0.152$, $p < 0.05$ | | $R = 0.281$, $R^2 = 0.079$, p = ns | |
| Variables | β | p | β | p | β | p | β | p | β | p |
| Age (yrs) | 0.075 | ns | −0.059 | ns | −0.021 | ns | −0.008 | ns | 0.060 | ns |
| Gender | 0.125 | ns | −0.142 | ns | −0.095 | ns | −0.336 | 0.001 | −0.218 | 0.030 |
| BMI | −0.048 | ns | 0.015 | ns | −0.040 | ns | 0.042 | ns | 0.036 | ns |
| SBP | 0.067 | ns | 0.114 | ns | 0.114 | ns | 0.042 | ns | 0.026 | ns |
| Smoking | −0.025 | ns | 0.109 | ns | 0.097 | ns | 0.028 | ns | 0.135 | ns |
| Sedentary lifestyle | −0.108 | ns | −0.064 | ns | −0.066 | ns | −0.130 | ns | −0.051 | ns |
| Glucose | −0.039 | ns | 0.043 | ns | 0.106 | ns | 0.040 | ns | 0.000 | ns |
| Total cholesterol | −0.157 | ns | −0.044 | ns | −0.101 | ns | 0.001 | ns | 0.062 | ns |
| Triglycerides | 0.219 | ns | 0.189 | ns | 0.139 | ns | 0.105 | ns | 0.058 | ns |
| **(a) O3I: Model 2** | $R = 0.320$, $R^2 = 0.102$, p = ns $\Delta R^2 = 0.036$, $p < 0.05$ | | $R = 0.373$, $R^2 = 0.139$, p = ns $\Delta R^2 = 0.028$, p = ns | | $R = 0.330$, $R^2 = 0.109$, p = ns $\Delta R^2 = 0.023$, p = ns | | $R = 0.433$, $R^2 = 0.187$, $p < 0.05$ $\Delta R^2 = 0.035$, $p < 0.05$ | | $R = 0.324$, $R^2 = 0.105$, p = ns $\Delta R^2 = 0.026$, p = ns | |
| Variables | β | p | β | p | β | p | β | p | β | p |
| Age (yrs) | 0.085 | ns | −0.051 | ns | −0.013 | ns | 0.002 | ns | 0.069 | ns |
| Gender | 0.162 | ns | −0.110 | ns | −0.066 | ns | −0.300 | 0.002 | −0.187 | ns |
| BMI | −0.003 | ns | 0.054 | ns | −0.004 | ns | 0.086 | ns | 0.074 | ns |
| SBP | 0.084 | ns | 0.128 | ns | 0.128 | ns | 0.059 | ns | 0.040 | ns |
| Smoking | −0.003 | ns | 0.128 | ns | 0.115 | ns | 0.050 | ns | 0.154 | ns |
| Sedentary lifestyle | −0.094 | ns | −0.052 | ns | −0.055 | ns | −0.117 | ns | −0.039 | ns |
| Glucose | −0.071 | ns | 0.016 | ns | 0.081 | ns | 0.009 | ns | −0.027 | ns |
| Total cholesterol | −0.104 | ns | 0.002 | ns | −0.058 | ns | 0.053 | ns | 0.106 | ns |
| Triglycerides | 0.176 | ns | 0.152 | ns | 0.105 | ns | 0.064 | ns | 0.022 | ns |
| O3I | −0.209 | 0.045 | −0.183 | ns | −0.168 | ns | −0.205 | 0.035 | −0.177 | ns |
| **(b) Total n-3PUFA: Model 2** | $R = 0.311$, $R^2 = 0.097$, p = ns $\Delta R^2 = 0.031$, p = ns | | $R = 0.397$, $R^2 = 0.158$, $p < 0.05$ $\Delta R^2 = 0.046$, $p < 0.05$ | | $R = 0.349$, $R^2 = 0.122$, p = ns $\Delta R^2 = 0.036$, $p < 0.05$ | | $R = 0.449$, $R^2 = 0.201$, $p < 0.01$ $\Delta R^2 = 0.049$, $p < 0.05$ | | $R = 0.321$, $R^2 = 0.103$, p = ns $\Delta R^2 = 0.024$, p = ns | |
| Variables | β | p | β | p | β | p | β | p | β | p |
| Age (yrs) | 0.092 | ns | −0.039 | ns | −0.003 | ns | 0.013 | ns | 0.075 | ns |
| Gender | 0.147 | ns | −0.116 | ns | −0.072 | ns | −0.309 | 0.001 | −0.199 | 0.047 |
| BMI | −0.012 | ns | 0.058 | ns | −0.002 | ns | 0.086 | ns | 0.067 | ns |
| SBP | 0.077 | ns | 0.125 | ns | 0.125 | ns | 0.054 | ns | 0.035 | ns |
| Smoking | −0.005 | ns | 0.134 | ns | 0.120 | ns | 0.054 | ns | 0.154 | ns |
| Sedentary lifestyle | −0.096 | ns | −0.049 | ns | −0.053 | ns | −0.115 | ns | −0.040 | ns |
| Glucose | −0.058 | ns | 0.021 | ns | 0.087 | ns | 0.017 | ns | −0.016 | ns |
| Total cholesterol | −0.086 | ns | 0.042 | ns | −0.024 | ns | 0.091 | ns | 0.125 | ns |
| Triglycerides | 0.170 | ns | 0.130 | ns | 0.086 | ns | 0.044 | ns | 0.015 | ns |
| Total n-3PUFA | −0.195 | ns | −0.238 | 0.018 | −0.212 | 0.038 | −0.246 | 0.012 | −0.173 | ns |

[a] Two block hierarchical multiple regression analysis was conducted. O3I: Omega-3 index = erythrocyte %EPA+%DPA of total erythrocyte fatty acids [50]. Total n-3PUFA = %EPA+%DPA+%DHA.

Similarly the proportion of participants with normal monocyte levels ($\leq 0.8 \times 10^9$/L) increased across increasing quartiles of O3I (80.6%, 93.5%, 93.8%, 100.0%, *p*-trend = 0.007; Fig. 2), with significant differences in the proportion of participants with monocyte levels $\leq 0.8 \times 10^9$/L found between the first (80.6%) and fourth quartiles (100%; $\chi^2 = 6.85$, p = 0.009). The proportion of participants with normal fibrinogen, WBC, neutrophil, lymphocyte, and eosinophil levels did not differ between O3I quartiles (data not shown). Basophils were not analysed because the basophil data for all participants were within the reference range.

## 4. Discussion

The aim of this cross sectional study was to determine whether erythrocyte n-3PUFA status, and in particular O3I, is a predictor of inflammation in older Australians. This study revealed a significant inverse association between O3I and the inflammatory mediators, CRP, WBC, neutrophils and monocytes. To date literature on the association between erythrocyte n-3PUFA levels, in particular O3I, and inflammation has been limited, therefore the results of this current study represent novel findings. Previous observational studies have generated conflicting results regarding the effect of dietary n-3PUFA intake on inflammatory biomarkers [11,13,35–40]. Prior studies have determined n-3PUFA levels using self-reported dietary intake, which is limited by recall bias and does not account for absorption or metabolism, or plasma n-3PUFA levels, which are a short term rather than long-term indicator of dietary intake [18,41].

An inverse association between O3I with CRP and IL-6 has previously been reported in a study of 992 predominantly male patients with coronary artery disease, aged 64 years and over [12]. Similarly, in a study of 357 healthy Yup'ik eskimos, there was an inverse association between erythrocyte DHA and EPA with CRP levels, however O3I was not reported in this study and several important confounders, such as physical activity and blood pressure, were not accounted for in the multivariate analysis [42]. Conversely, a study of healthy Italian participants aged 20–98 years, did not find an association between erythrocyte n-3PUFA levels and CRP [10]. However, the levels of CRP and erythrocyte EPA and DHA reported were low, which may have attenuated any potential relationship.

The O3I values in the current study were higher than those

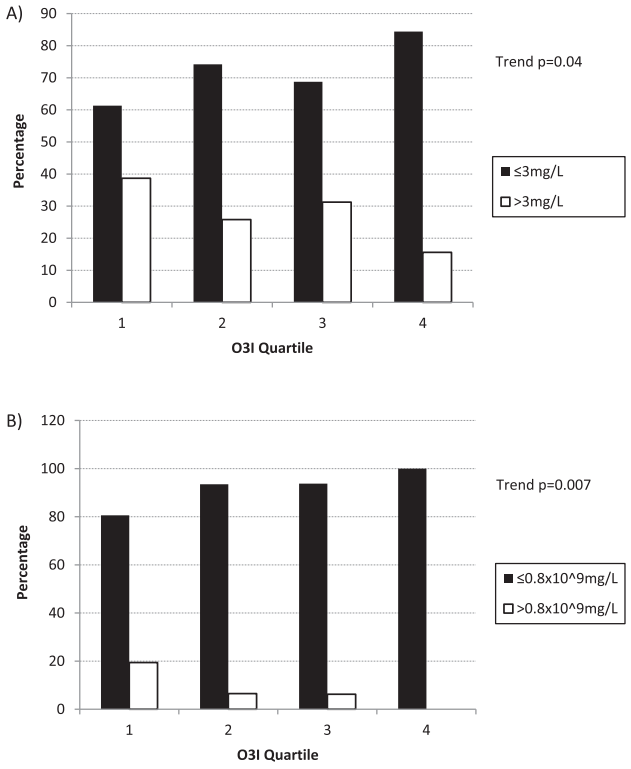


**Fig. 2.** The percentage of older adults (aged ≥65yrs) with CRP levels or monocyte counts within reference ranges for each O3I quartile. A. The percentage of participants within each O3I quartile with CRP levels ≤3.0 mg/L or >3.1 mg/L [30]. B. The percentage of participants within each O3I quartile with monocyte count ≤0.8 × $10^9$/L or >0.8 × $10^9$/L [31].

reported in other comparable literature [12,19,20]. The age of the study population may have contributed to the higher overall O3I mean. A cross sectional study of 160,000 American participants found that O3I can increase by as much as 7% per decade up until 70 years [43]. Variations in the methodologies used to analyse erythrocyte membrane fatty acids may also result in different mean values between studies [44]. Regardless, the relationships and trends found would be comparable.

The results of this study may have also been influenced by the BMI of participants (27.0 ± 4.0 kg/$m^2$). BMI was adjusted for within the multivariate regression analyses, however, the percentage of obese participants within this study population was relatively low. Le et al. found in a systematic review and meta-analysis of RCTs that n-3PUFA reduce plasma CRP in subjects with BMI <30 kg/$m^2$ but not in subjects with BMI >30 kg/$m^2$ [17]. Therefore the lower BMI of this study population may have contributed to the inverse correlation found. It has been proposed that the lack of association between CRP levels and n-3PUFA levels in obese participants is due to increased levels of the n-6PUFA, arachidonic acid, in adipose tissue. N-6PUFA are associated with the production of pro-inflammatory cytokines [45]. Future studies assessing the association between n-3PUFA and CRP within obese populations are warranted [46].

WBC are widely recognised as reliable biomarkers of inflammation, however have not received the same attention as CRP with respect to their relationship with n-3PUFA or their role in the relationship between n-3PUFA levels and inflammation [47]. To our knowledge, this is the first study to explore the relationship between n-3PUFA levels and WBC and the WBC subgroups. Both O3I and total n-3PUFA were inversely associated with WBC, monocyte and neutrophil cell counts independently, and with monocytes when corrected for potential confounders. Total n-3PUFA was also inversely related to WBC, neutrophils and monocytes following multivariate adjustment for confounding variables. In addition, we found inverse associations between DPA levels and WBC and neutrophil counts. Since dietary intake is poorly correlated with DPA levels, it is believed that DPA is predominately produced endogenously from the elongation of EPA or retroconversion of DHA [48]. Most observational studies have explored alterations in WBC and WBC subgroup counts in relation to adherence to a Mediterranean diet, and have shown a reduction in WBC, monocyte and neutrophil count [49–51]. However this association cannot be attributed to n-3PUFA levels alone as many other factors may contribute to the health benefits of a Mediterranean diet including polyphenol, olive oil and legume intake [52,53]. Neutrophils and monocytes are the first WBC involved in the inflammatory response. Our finding that higher levels of O3I and total n-3PUFA are associated with reduced monocyte counts suggests that increased intake of n-3PUFA may reduce the proliferation of inflammatory WBC components [54].

This study did not detect an association between O3I and fibrinogen. Low fibrinogen levels (3.5 g/L ± 0.6) within this study population may be a likely reason for this lack of association. Participants reported low levels of smoking and sedentary behaviour, two factors that when elevated are associated with increasing fibrinogen levels, and had high HDL levels which is associated with lower fibrinogen levels [7,8]. Similarly Kalogeroulous et al. did not find an association between O3I and fibrinogen in healthy Greek male and female participants with fibrinogen levels of 3.18 g/L and 3.14 g/L, respectively [13]. These findings support the postulate that overall healthy lifestyle and dietary behaviours may have attenuated the potential benefits from higher n-3PUFA levels. Conversely, in RCTs conducted in populations with elevated fibrinogen levels, supplementation has resulted in reductions in fibrinogen levels of 10.6%–30.0% [55,56]. Similarly, an inverse association was found between n-3PUFA levels and fibrinogen in healthy cigarette smoking males [57]. The results of the aforementioned studies together with those of the current study suggest that the benefits of n-3PUFA in regards to reducing fibrinogen levels may be most pronounced in those with elevated fibrinogen levels or in those with lifestyle factors known to increase levels.

The current study had several strengths including the use of an objective biomarker of long term dietary n-3PUFA intake rather than dietary n-3PUFA intake alone. Dietary intake assessment is known to have inherent measurement errors and bias [39]. The relatively large sample and comprehensive data collection enabled the identification and analysis of potential confounding variables such as smoking status, sedentary lifestyle, blood pressure, BMI, fasting glucose levels, triglycerides and total cholesterol. Furthermore, as participants consuming n-3PUFA supplements were excluded, findings were based on physiological erythrocyte concentrations; which provides a more objective measure of long term PUFA exposure, taking into account intake and metabolism [18]. There are also some limitations, including the use of self-report medical history and food frequency questionnaires which may be subject to recall bias. In addition, although the study accounted for many potential variables, we cannot exclude entirely that other lifestyle or dietary patterns have not contributed to the aforementioned associations. Finally, as this study was a cross-sectional analysis, findings from this study cannot suggest causal inverse relationships between n-3PUFA levels and inflammatory biomarkers. Moreover, plasma samples were not available to measure additional markers of inflammation such as IL-6 and TNFα, this remains a weakness of the current study.

In conclusion we found a significant independent inverse

association between erythrocyte n-3PUFA and circulating inflammatory biomarkers CRP, WBC, neutrophils and monocytes in a cross-sectional analysis of older Australians. The inverse association between O3I and monocytes, and between total n-3PUFA and WBC, neutrophils and monocytes, persisted after multivariate adjustments for potential confounders. Future studies are required to explore the potential anti-inflammatory effects of erythrocyte n-3PUFA levels in other populations, including younger and overweight and obese adults.

## Author contributions

MLG MV and ML designed research; SN KK and MO conducted research; MO analysed data; MO, MLG, LMW, SN and KK wrote the paper; MO had primary responsibility for final content; and all authors contributed to the critical revision of the manuscript and approved the final manuscript.

## Conflict of interest

None of the authors had a financial or personal conflict of interest.

## Acknowledgements

The research on which this paper is based was conducted as part of the Retirement Health and Lifestyle Study, The University of Newcastle. We are grateful to the Australian Research Council (ARC Linkage Project Grant LP0883378), Central Coast Local Health District Public Health Unit, UnitingCare Ageing NSW.ACT, Urbis Pty Ltd, Valhalla Village Pty Ltd and Hunter Valley Research Foundation for funding the initial study and to the men and women of the Central Coast region who provided the information recorded. The authors wish to acknowledge the involvement of the members of the RHLS Group. The authors would also like to acknowledge Ms Melissa Fry for her assistance with the fatty acid analysis.

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