Journal of Nutrition & Intermediary Metabolism 5 (2016) 78–85

Contents lists available at ScienceDirect

Journal of Nutrition & Intermediary Metabolism

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

Association between erythrocyte omega-3 polyunsaturated fatty acid levels and fatty liver index in older people is sex dependent

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
27 April 2016
Accepted 28 April 2016
Available online 4 May 2016

Keywords:
n-3polyunsaturated fatty acids
Omega-3 index
Non-alcoholic fatty liver disease
Fatty liver index
Older adults
Metabolic syndrome

ABSTRACT

Background/Objectives: Non-alcoholic fatty liver disease (NAFLD) is highly prevalent in older people but currently no specific drugs are available for its treatment. Omega-3 polyunsaturated fatty acids (n-3PUFA), known for their lipid-lowering, anti-inflammatory and anti-hypertensive properties, may have therapeutic potential for the management of NAFLD. The aim of this study was to determine whether n-3PUFA levels are associated with the prevalence of NAFLD in older adults.

Methods: A cross-sectional sample of older adults aged 65–95 years (n = 620) from the Retirement Health and Lifestyle Study (RHLS) was analysed. Fatty Liver Index (FLI) scores, used as an indicator of NAFLD risk, were calculated using a validated algorithm that incorporates body mass index, waist circumference, plasma triglycerides and γ-glutamyl transferase. Omega-3 index scores (O3I, %eicosapentaenoic acid plus %docosahexaenoic acid) were determined by analysing the fatty acid composition of erythrocyte membranes by gas chromatography.

Results: Following application of exclusion criteria, 475 participants were included in the analysis (age 77.9 ± 7.0 years; 60.4% females). Of these, 216 participants had FLI scores (≥60) suggestive of NAFLD (age 77.0 ± 6.6 years; 49.1% females). O3I was significantly lower in participants with NAFLD compared to those without NAFLD ($p < 0.01$). A significant inverse relationship was found between O3I and FLI ($r = -0.165$; $p < 0.001$). This relationship was gender specific with women, but not men, showing a significant association ($r = -0.206$; $p < 0.001$).

Conclusions: The current study demonstrated a sex-dependent inverse relationship between erythrocyte n-3PUFA concentrations and NAFLD in older adults. The finding supports the proposal for sex-stratified n-3PUFA intervention trials in this high-risk age group.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), defined as the accumulation of fat (>5%) in the hepatocytes of the liver [1–3], represents a broad spectrum of conditions ranging from steatosis to the more severe non-alcoholic steatohepatitis (NASH) [3]. If left undetected or untreated, NAFLD can progress to fibrosis, cirrhosis and potentially liver failure [4]. In 2012, NAFLD was reported to be the most prevalent of all the liver diseases in Australia and was estimated to affect 5.5 million Australians including approximately 40% of adults aged 50 years or older [5]. By the year 2030, the number of Australians diagnosed with NAFLD is expected to exceed 7 million [5]. Furthermore, it has also been reported that NAFLD is becoming more prevalent in the older population (>60 years) [6] with post-menopausal women more likely to develop NAFLD than men of the same age [6].

Recent studies suggest that NAFLD is the hepatic expression of the metabolic syndrome with risk factors including insulin resistance, obesity, hyperlipidaemia, and hypertension [7–10]. Two stages have been proposed in the pathogenesis of the disease [1,6,11,12]. The first stage is the accumulation of free fatty acids (FFA) and triglycerides (TG) in the liver due to excessive storage of

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http://dx.doi.org/10.1016/j.jnim.2016.04.007

fat in the adipose tissue [13] and is associated with high dietary fat/sugar intake or *de-novo* lipogenesis [13]. The second is mitochondrial dysfunction, oxidative stress and inflammation in addition to steatosis and results from the increase in serum FFA and serum TG [11]. However, not all patients with steatosis progress to NASH, indicating that additional stages may be involved in disease progression [14,15].

Currently, there is no specific treatment for NAFLD. Management options include lifestyle modifications (dietary energy restrictions, increased physical activity and weight loss) [16,17] and pharmacological agents (anti-hypertensive, lipid lowering, insulin sensitizing and weight loss medications) [14,15]. However, these options are complicated by the need for long-term compliance and concerns regarding the safety of medications such as insulin-sensitizing agents [14]. Development of safe and efficacious treatments and prevention strategies is therefore highly desirable [15].

A promising therapy for the management and treatment of NAFLD is increasing omega-3 polyunsaturated fatty acids intake (n-3PUFA) [3]. N-3PUFA are recognised to have lipid-lowering, anti-inflammatory [18] and anti-hypertensive properties, although the mechanisms underlying these effects have not been fully elucidated. Evidence suggests that eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids may reduce serum and hepatic TG, and decrease the production of the pro-inflammatory cytokines tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6) [19]. Other studies have also shown that n-3PUFA compete with omega-6 fatty acids for metabolism, thereby promoting the production of less-inflammatory eicosanoids (thromboxane and prostaglandins of the 3-series), and reducing the formation of pro-inflammatory 2-series eicosanoids [20]. In addition, EPA and DHA may serve as precursors for the synthesis of resolvins and protectins, promoting resolution of the inflammatory component of NAFLD [17]. Observational and interventional studies have reported an inverse association between n-3PUFA and NAFLD. Previous studies have been conducted in younger populations using objective measures of NAFLD [3]. Further work is needed to establish the relationship between long-term n-3PUFA status and NAFLD.

Typically, imaging procedures, such as ultrasound, computerized tomography scans and magnetic resonance imaging, have been used to diagnose fatty liver disease. However, an alternative validated non-invasive method for diagnosing NAFLD is the Fatty Liver Index (FLI) [21–23]. This index assesses risk of fatty liver disease using an algorithm based on four criteria; body mass index (BMI), TG, waist circumference and γ-glutamyl transferase (GGT) [18]. Our study used the FLI to assess NAFLD risk in a group of adults aged 65 years or older. While previous studies have been conducted with younger populations using non-FLI diagnostic tools [19,24,25], the current study was the first to examine the potential relationship between n-3PUFA status and NAFLD in older Australians. The omega-3 index (O3I), the sum of %EPA and %DHA in erythrocyte membranes [26], was used as a validated measure of long-term n-3PUFA status.

2. Subjects and methods

2.1. Subjects and study design

A cross-sectional analysis was conducted using data collected for the Retirement Health and Lifestyle Study (RHLS) between 2010 and 2012. The RHLS eligibility criteria included: $\geq$65 years of age, living independently in a retirement or community dwelling for 12 months or more and living within the Gosford/Wyong Local Government Areas. People were ineligible for the RHLS if: they were not independently living or were residing in a communal setting other than a retirement village, their listed address was not their primary residence, or another member of their household was taking part in the study. People with language and/or other communicative difficulties, or who were cognitively impaired and/or unable to provide informed consent, were also excluded. Participants were included in the current analyses if they had erythrocyte samples available for fatty acid analysis, valid height, weight, waist circumference, GGT and TG measures, and their liver disease status and daily alcohol intake could be determined. Participants were excluded if they reported liver disease other than fatty liver disease, or an alcohol intake exceeding 20.5 g per day.

All RHLS participants (n = 831) took part in an interviewer-administered questionnaire (IAQ) that collected information relating to demographics and health status including lifestyle factors and physical activity [27]. A subset of participants also took part in a clinical assessment that included measurement of anthropomorphic characteristics and sitting blood pressures. The majority of clinic participants provided a fasted blood sample and completed a self-administered food frequency questionnaire (FFQ) [28] as part of a physical activity questionnaire [29–32]. Erythrocyte samples were available for determination of O3I for 620 participants.

The study protocols were approved by the University of Newcastle Human Research Ethics Committee (Reference No. H-2008-0431) and the Northern Sydney Central Coast Health Human Research Ethics Committee (Reference No. 1001-031 M) and all participants provided written informed consent.

2.2. Anthropometric measures

Anthropometric measurements were taken by trained research officers using standardised protocols outlined by the World Health Organisation [33]. Participants were weighed in light clothing and without shoes to the nearest 100 g using standardised digital scales (Tanita HD-316 scales, Tanita Corporation, Tokyo, Japan; or Wedderburn UWPM150 Digital Platform Scales, Wedderburn Scales, Australia). Height was measured without shoes to the nearest 0.1 cm using a portable stadiometer (Design No. 1013522, Surgical and Medical Products, Seven Hills, NSW, Australia). Waist circumference was measured between the lower costal margin and the iliac crest using a non-elastic flexible measuring tape (150 cm $\times$ 12 mm, Sullivans International), to the nearest 0.1 cm. Subjects were asked to remove any heavy clothing and belts, and measurements were conducted either on bare skin or over loose fitting clothing. Hip measurements, to the nearest 0.1 cm, were taken from the greatest posterior protuberance of the buttocks using a non-elastic flexible measuring tape. All anthropometric measurements were taken twice. If the measurements disagreed by more than set tolerance limits (weight, 800 g; height, 1 cm; waist circumference, 2 cm; and hip circumference, 2 cm) 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. Height and weight measurements were used to calculate BMI (kg/m^2).

2.3. Sitting blood pressure and pulse measures

Sitting blood pressure measurements were conducted by trained research officers following protocols outlined by the National Heart Foundation of Australia [34]. Participants were asked to avoid strenuous exercise for 24 h prior to measurement, fast overnight and abstain from smoking on the morning of measurement. Two measurements were taken at 1 min intervals using an Omron 1A2 digital automatic blood pressure monitor (Omron, Australia) with participants seated in a chair, feet flat on the ground and legs uncrossed. If the two readings differed by more than

10 mmHg or 6 mmHg for the systolic and diastolic blood pressures, respectively, up to two further measurements were taken until consecutive readings did not vary by greater than these amounts.

2.4. Biochemical analyses

Blood samples were collected by trained phlebotomists and were analysed by Hunter Area Pathology Service (HAPS). Routine biochemical analyses included blood lipids (total cholesterol (TC), LDL-cholesterol, HDL-cholesterol, TG), diabetes markers (fasting blood glucose, insulin), inflammatory marker (C-reactive protein (CRP)), and liver function tests (total bilirubin, GGT, alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate aminotransferase (AST)).

For erythrocyte membrane fatty acid composition determination, blood samples were centrifuged at 3000×g for 10 min to separate the plasma and erythrocyte fractions. The fatty acid composition of the erythrocyte membranes was then determined via direct trans-esterification of the washed erythrocyte fractions [35], followed by gas chromatographic analysis [36]. A known fatty acid mixture was used to identify peaks according to retention time and their concentration was determined using a Hewlett Packard 6890 Series GC with Chemstation Version A. 04.02.

2.5. Dietary assessments

Dietary intakes of energy, protein, carbohydrate, fat and alcohol were determined using a 41-question self-administered food frequency questionnaire (FFQ) that was adapted from the validated CSIRO FFQ [28], and modified for self-administration. Nutrient intakes were analysed in FoodWorks Professional Edition Version 6.0.2562 (Xyris Software, Brisbane, Queensland, Australia). Australian databases used included Abbott products, AusFoods (Brands) 2006, AusNut (All Foods) 2007, Australia (Fatty Acids), and the New Zealand vitamin and mineral supplements 1999.

2.6. Fatty liver index

FLI was calculated using an algorithm based on four markers: BMI, TG, GGT, and waist circumference [21]. Evidence shows that this index detects fatty liver with an accuracy of 0.84 [21]. The index is scored 0–100; a score of less than 30 rules out fatty liver whilst a score of 60 or over indicates fatty liver [18–20]. The algorithm used to calculate the FLI was: $[e^{0.953 \times \text{loge(triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{loge(GGT)} + 0.053 \times \text{waist circumference} - 15.745}]/[1 + e^{0.953 \times \text{loge(triglycerides)} + 0.139 \times \text{BMI} + 0.718 \times \text{loge(GGT)} + 0.053 \times \text{waist circumference} - 15.745}] \times 100$ [21].

2.7. Statistical analysis

Data was analysed using Statistical Package for the Social Sciences (SPSS; Release 22.0, Chicago, IL: SPSS Inc.). Data was reported as mean ± standard deviation or frequency, as appropriate. Groups were compared using chi-square analyses and independent sample two-tailed t-tests. Differences in the proportion of people with NAFLD across O3I quartiles were reported as odds ratios (OR, 95% CI) using the lowest quartile as the reference. Bivariate and multivariate relationships were analysed using Pearson product-moment correlation and hierarchical regression analyses. Variables were entered into the hierarchical regression in three blocks. Demographic variables (age and gender) were entered into the regression analysis at block 1. Clinically relevant biochemical variables that correlated with FLI (HDL, CRP, fasting glucose and insulin) were entered at block 2 and the test variable, O3I, was entered at block 3. Pair-wise exclusion for missing data was employed in all regression analyses. Statistical significance was set at $p < 0.05$.

3. Results

Following application of the exclusion criteria, 475 participants were included in the analyses (age, 77.9 ± 7.0 years; 60.4% females). Fatty liver index scores were calculated and participants were stratified on the basis of NAFLD status. Two hundred and sixteen participants (45.5%) had FLI scores ≥60 and were classified as having NAFLD. The remaining participants (n = 259, 54.5%) had FLI scores of <60 and were classified as non-NAFLD. Participants' demographic characteristics are outlined in Table 1, together with the results of anthropometric, blood pressure and blood evaluations. Participants with NAFLD were younger (77.0 ± 6.6 years) than those without NAFLD (78.6 ± 7.3 years) and the proportion of females was lower in the NAFLD group (49.1% vs 70.4%).

As expected, the mean Fatty Liver Index, and the levels of the four criterion variables, BMI, waist circumference, GGT and TG were significantly higher in NAFLD participants compared to non-NAFLD participants (Table 1). Participants with NAFLD had significantly higher body weight, BMI, waist circumference and waist-hip ratio than non-NAFLD. When the NAFLD and non-NAFLD groups were stratified by gender it was found that the mean waist circumferences of all four subgroups were above the National Heart Foundation reference cut-offs for increased chronic disease risk. The elevations were more pronounced in females. The mean waist circumferences for NAFLD females and non-NAFLD females were 31.9% and 8.3% above the reference cutoff of 80 cm, whereas the mean waist circumference of NAFLD males and non-NAFLD males were 18.1% and 0.04% above the cutoff of 94 cm [34,37]. Participants with NAFLD also had significantly higher systolic and diastolic blood pressures, fasting glucose, insulin, ALT and CRP, and significantly lower HDL and LDL, compared to those without NAFLD. Similar differences were observed between NAFLD and non-NAFLD participants when the male and female subgroups were analysed separately; although some differences were no longer statistically significant.

3.1. Nutrient intake of study participants

Participants' nutrient intakes are summarised in Table 2. No significant differences in energy, carbohydrate or protein intake were detected between participants with NAFLD and those without NAFLD. Total fat, polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) intake were lower in participants with NAFLD, but only the differences in PUFA intake observed in the-all participant and female-only comparisons were statistically significant. Alcohol intake was higher in participants with NAFLD than those without NAFLD (5.4 ± 6.3 g/d versus 4.2 ± 5.7 g/d), and higher among men compared to women regardless of NAFLD status ($p < 0.05$).

3.2. Erythrocyte membrane fatty acid compositions

The fatty acid composition of erythrocyte membranes is presented in Table 3. Erythrocyte membrane concentrations of palmitic acid (16:0) and total saturated fatty acids were significantly reduced, and palmitoleic acid (16:1n-7) significantly increased, in NAFLD compared to non-NAFLD participants. Similar differences were evident when participants were stratified on gender and then NAFLD status, however, with the exception of the higher palmitoleic acid content, these differences failed to achieve statistical significance. Linoleic acid (18:2n-6) and α-linolenic acid (18:3n-6) were the only n-6PUFA found to be significantly different between NAFLD and non-NAFLD participants (Table 3). EPA (20:5n-3) was

Table 1
Demographic, anthropometric, blood pressure and blood analyses measures for participants grouped by gender and NAFLD status.[a]

	All participants		Males		Females	
	Non-NAFLD (n = 259)	NAFLD[b] (n = 216)	Non-NAFLD (n = 78)	NAFLD (n = 110)	Non-NAFLD (n = 181)	NAFLD (n = 106)
Age (yrs)	78.6 ± 7.3	77.0 ± 6.6*	78.7 ± 7.2	76.7 ± 6.3	78.6 ± 7.4	77.4 ± 6.8
Anthropometric measurements, blood pressure and pulse						
Height (m)	1.6 ± 0.1	1.6 ± 0.1***	1.7 ± 0.1	1.7 ± 0.1*	1.6 ± 0.1	1.6 ± 0.1
Weight (kg)	65.5 ± 10.0	86.4 ± 12.5***	71.4 ± 9.4	90.6 ± 12.4***	62.9 ± 9.1	81.9 ± 11.0***
BMI (kg/m[b])	25.4 ± 3.1	32.0 ± 4.0***	25.0 ± 2.6	30.8 ± 3.4***	25.6 ± 3.3	33.3 ± 4.2***
Waist (cm)	89.0 ± 8.8	108.3 ± 9.6***	94.4 ± 8.2	111.0 ± 9.1***	86.6 ± 8.1	105.5 ± 9.3***
Hip (cm)	103.2 ± 7.8	115.5 ± 9.1***	101.8 ± 5.7	111.7 ± 6.7***	103.7 ± 8.5	119.4 ± 9.5***
Waist/Hip Ratio	0.86 ± 0.07	0.94 ± 0.08***	0.93 ± 0.07	0.99 ± 0.06***	0.84 ± 0.06	0.88 ± 0.05***
SBP (mmHg)	144.9 ± 21.1	151.2 ± 19.3**	146.0 ± 22.1	154.7 ± 18.4**	144.4 ± 20.7	147.6 ± 19.6
DBP (mmHg)	72.8 ± 9.4	76.1 ± 10.5***	73.4 ± 9.3	77.8 ± 10.1**	72.6 ± 9.4	74.5 ± 10.8
Pulse (mmHg)	67.5 ± 10.5	67.8 ± 11.8	64.3 ± 9.7	66.4 ± 12.7	68.9 ± 10.5	69.3 ± 10.7
Glycaemic Indices						
Glucose (mmol/L)	5.3 ± 0.8	5.9 ± 1.2***	5.4 ± 0.8	6.1 ± 1.3**	5.2 ± 0.7	5.8 ± 1.1***
Insulin (mIU/L)	5.9 ± 3.7	10.8 ± 7.0***	5.8 ± 3.5	9.9 ± 6.5***	5.9 ± 3.7	11.8 ± 7.4***
Liver Function Tests						
GGT (U/L)	26.7 ± 14.0	45.0 ± 43.9***	26.6 ± 12.7	47.3 ± 50.2***	26.7 ± 14.5	42.6 ± 36.2***
ALT (U/L)	20.2 ± 9.1	24.0 ± 11.4***	20.6 ± 7.8	25.7 ± 11.9**	20.1 ± 9.6	22.2 ± 10.5
AST (U/L)	19.4 ± 6.3	19.4 ± 8.0	18.8 ± 6.0	19.7 ± 7.9	19.6 ± 6.4	19.1 ± 8.1
CRP (mg/L)	2.7 ± 3.2	4.0 ± 5.4**	2.5 ± 2.9	3.3 ± 4.7	2.8 ± 3.3	4.8 ± 6.0**
Lipids						
Cholesterol (mmol/L)	4.6 ± 1.1	4.4 ± 1.0	4.1 ± 0.9	4.2 ± 0.9	4.8 ± 1.0	4.7 ± 1.1
TG (mmol/L)	1.1 ± 0.4	1.7 ± 0.8***	1.0 ± 0.4	1.6 ± 0.8***	1.1 ± 0.4	1.7 ± 0.8***
HDL (mmol/L)	1.6 ± 0.4	1.3 ± 0.4***	1.4 ± 0.4	1.2 ± 0.3**	1.7 ± 0.4	1.4 ± 0.4***
LDL (mmol/L)	2.5 ± 0.9	2.3 ± 0.9*	2.3 ± 0.9	2.2 ± 0.8	2.6 ± 0.9	2.4 ± 1.0
Fatty Liver Index	31.6 ± 16.6	80.8 ± 11.1***	34.3 ± 15.3	80.3 ± 11.1***	30.5 ± 17.0	81.3 ± 11.1***

*Statistically significant at $p < 0.05$; ** Statistically significant at $p < 0.01$; *** Statistically significant at $p < 0.001$.
[a] Data presented as Mean ± SD. Group differences between NAFLD and non-NAFLD participants were assessed using independent samples t-tests (2-tailed).
[b] Participants were defined as having NAFLD if they had a FLI score ≥60 [21]. FLI score includes BMI, waist circumference, TG and GGT [21].

significantly lower in participants with NAFLD ($p < 0.05$). However after adjusting for gender, no significant differences in EPA levels were found. DHA (22:6n-3), total n-3PUFA, and O3I were significantly lower in participants with NAFLD compared to those without NAFLD in both the all participants and female-only subgroups, but no differences were noted in males (Table 3).

3.3. Correlation analyses

Bivariate correlation analyses showed that O3I was negatively associated with weight, BMI, waist circumference, waist:hip ratio, glucose, and TG, and positively correlated with HDL in all participants. Gender specific differences were evident (Table 4). In the female-only group, O3I was inversely associated with weight, BMI, waist circumference, hip circumference, TG, and CRP and positively associated with AST, ALT and HDL. These associations did not exist in males. O3I and FLI were inversely associated in all participants, however, stratification on gender revealed that this relationship was also specific to females. O3I was also inversely associated with total MUFA and total n-6PUFA, and positively associated with total n-3PUFA, regardless of gender.

3.4. O3I and NAFLD

The prevalence of NAFLD among participants sub-grouped into quartiles on the basis of their O3I scores is presented in Table 5. When NAFLD prevalence was compared across the four quartiles, the odds of having NAFLD was lower in participants in the highest O3I quartile (0.65) compared to those in the lowest O3I quartile (1.0; *P*-trend = 0.018). This trend was also apparent in females, with the odds ratio decreasing from 1.0 (quartile 1) to 0.55 (quartile 4; *P*-trend = 0.025), but not in males, where the odds ratios for the first and fourth quartiles were 1.00 and 1.09, respectively.

It was noted that the prevalence and risk of NAFLD for the lower two O3I quartiles were comparable in magnitude (Quartile 1: 49.6%, OR = 1.00; Quartile 2: 55.1%, OR = 1.25) as was the

Table 2
Nutrient intakes of participants grouped by gender and NAFLD status.[a]

	All participants		Males		Females	
	Non-NAFLD (n = 259)	NAFLD[b] (n = 216)	Non-NAFLD (n = 78)	NAFLD (n = 110)	Non-NAFLD (n = 181)	NAFLD (n = 106)
Energy (kJ)	7840.9 ± 3164.2	7877.7 ± 2926.3	7833.1 ± 2521.3	8107.1 ± 3217.4	7844.3 ± 3410.5	7639.7 ± 2583.7
Protein (g)	87.6 ± 38.5	88.1 ± 33.4	86.7 ± 31.6	90.3 ± 35.2	88.0 ± 41.2	85.8 ± 31.4
Total Fat (g)	67.6 ± 34.2	64.8 ± 29.7	66.6 ± 28.8	66.0 ± 31.7	68.0 ± 36.3	63.5 ± 27.5
SFA (g)	22.9 ± 11.9	22.8 ± 11.3	22.6 ± 10.4	23.1 ± 11.9	23.0 ± 12.5	22.5 ± 10.7
MUFA (g)	26.2 ± 14.0	25.3 ± 13.0	26.0 ± 12.3	26.0 ± 13.9	26.2 ± 14.7	24.6 ± 12.1
PUFA (g)	11.9 ± 9.9	10.2 ± 6.1*	11.3 ± 7.1	10.3 ± 6.6	12.2 ± 10.9	10.0 ± 5.5*
Carbohydrate (g)	207.8 ± 92.2	213.5 ± 91.0	208.5 ± 72.2	219.6 ± 100.4	207.5 ± 99.8	207.3 ± 80.2
Fibre (g)	30.9 ± 15.4	30.9 ± 14.5	29.9 ± 12.1	31.4 ± 15.1	31.4 ± 16.7	30.4 ± 13.9
Cholesterol (mg)	233.9 ± 131.1	236.8 ± 112.9	224.8 ± 106.8	243.8 ± 121.4	237.9 ± 140.4	229.5 ± 103.4
Alcohol (g)	4.2 ± 5.7	5.4 ± 6.3*	5.7 ± 6.6	7.0 ± 6.8	3.5 ± 5.2	3.8 ± 5.2

* Statistically significant at $p < 0.05$.
[a] Data presented as Mean ± SD. Group differences between NAFLD and non-NAFLD participants were assessed using independent samples t-tests (2-tailed).
[b] Participants were defined as having NAFLD if they had a FLI score ≥60 [21]. FLI score includes BMI, waist circumference, TG and GGT [21].

Table 3
Erythrocyte membrane fatty acid composition of participants grouped by gender and NAFLD status.[a]

Fatty acid (%)	All participants		Males		Females	
	Non-NAFLD (n = 259)	NAFLD[b] (n = 216)	Non-NAFLD (n = 78)	NAFLD (n = 110)	Non-NAFLD (n = 181)	NAFLD (n = 106)
SFA						
16:0	22.9 ± 1.1	22.7 ± 1.3*	22.9 ± 1.1	22.7 ± 1.4	22.9 ± 1.1	22.6 ± 1.3
18:0	18.6 ± 1.2	18.4 ± 1.6	18.7 ± 1.4	18.3 ± 1.6	18.6 ± 1.1	18.5 ± 1.5
20:0	0.6 ± 0.1	0.6 ± 0.4	0.6 ± 0.1	0.6 ± 0.6	0.6 ± 0.1	0.6 ± 0.1
Total SFA	42.2 ± 1.8	41.7 ± 2.4*	42.2 ± 2.1	41.7 ± 2.5	42.1 ± 1.7	41.8 ± 2.4
MUFA						
16:1n-7	0.5 ± 0.2	0.6 ± 0.3***	0.5 ± 0.2	0.6 ± 0.3*	0.6 ± 0.2	0.7 ± 0.4***
18:1n-7	1.8 ± 0.3	1.7 ± 0.4	1.8 ± 0.3	1.7 ± 0.4*	1.8 ± 0.3	1.8 ± 0.4
Total MUFA	2.3 ± 0.5	2.3 ± 0.6	2.2 ± 0.5	2.2 ± 0.6	2.3 ± 0.5	2.5 ± 0.7
n-6 PUFA						
18:2n-6	9.0 ± 1.4	8.5 ± 1.5**	9.2 ± 1.3	8.7 ± 1.5*	8.9 ± 1.4	8.4 ± 1.5**
18:3n-6	0.2 ± 0.2	0.2 ± 0.2*	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2*
20:2n-6	0.3 ± 0.1	0.3 ± 0.3	0.3 ± 0.1	0.3 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
20:3n-6	1.5 ± 1.2	1.5 ± 1.1	1.5 ± 1.2	1.6 ± 1.2	1.4 ± 1.1	1.4 ± 1.1
20:4n-6	16.7 ± 2.5	16.9 ± 2.4	16.9 ± 2.6	16.9 ± 2.4	16.6 ± 2.5	16.8 ± 2.4
Total n-6PUFA	27.7 ± 2.6	27.5 ± 2.5	28.1 ± 2.7	27.7 ± 2.5	27.5 ± 2.6	27.2 ± 2.6
n-3 PUFA						
18:3n-3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:5n-3	1.7 ± 1.0	1.6 ± 0.9*	1.5 ± 0.9	1.5 ± 0.8	1.8 ± 1.0	1.7 ± 0.9
22:5n-3	3.6 ± 0.7	3.5 ± 0.7	3.6 ± 0.7	3.5 ± 0.6	3.6 ± 0.7	3.5 ± 0.8
22:6n-3	6.9 ± 1.4	6.6 ± 1.3**	6.5 ± 1.5	6.5 ± 1.4	7.1 ± 1.4	6.6 ± 1.3**
Total n-3PUFA	12.5 ± 2.6	11.9 ± 2.4**	11.9 ± 2.5	11.7 ± 2.4	12.7 ± 2.6	12.0 ± 2.4*
Omega-3 Index[c]	8.6 ± 2.2	8.1 ± 2.0**	8.0 ± 2.1	8.0 ± 2.0	8.9 ± 2.2	8.3 ± 2.0*

* Statistically significant at $p < 0.05$; ** Statistically significant at $p < 0.01$; *** Statistically significant at $p < 0.001$.
[a] Data presented as Mean ± SD. Group differences between NAFLD and non-NAFLD participants were assessed using independent samples t-tests (2-tailed).
[b] Participants were defined as having NAFLD if they had a FLI score ≥60 [21]. FLI score includes BMI, waist circumference, TG and GGT [21].
[c] O3I: Omega-3 index = erythrocyte EPA% + DPA% of total erythrocyte fatty acids [50].

prevalence and risk of NAFLD in the upper two O3I quartiles (Quartile 3: 38.3%, OR = 0.63; Quartile 4: 39.0%, OR = 0.65). When the prevalence of NAFLD among participants in the lower two quartiles was combined (n = 124, 52.3%, OR = 1.00) and compared to the prevalence of NAFLD among participants in the upper two quartiles (n = 92, 38.7%, OR = 0.57), a significant reduction in the risk of NAFLD was observed in the quartiles with the higher O3I scores ($\chi^2 = 8.94$, p = 0.003).

3.5. Hierarchical multiple linear regression analysis

The results of the hierarchical regression analysis of the relationship between the fatty liver index, O3I scores and other clinically relevant variables are outlined in Table 6. Age and gender were significantly associated with FLI (Model 1, R = 0.239, $p < 0.001$) and accounted for 5.7% of variation in FLI scores ($\Delta R^2 = 0.057$, $p < 0.001$). The addition of fasting glucose, insulin, HDL and CRP at

Table 4
Correlation analyses of the relationship between omega-3 fatty acids and age, anthropometric measures, and blood biochemistry for all participants and for participants grouped by gender.[a]

	All participants (n = 475)	Males (n = 188)	Females (n = 287)
Omega-3 index[b]			
Height (m)	−0.058	0.052	0.107
Weight (kg)	−0.120**	0.037	−0.126*
BMI (kg/m2)	−0.103*	0.023	−0.171**
Waist (cm)	−0.139**	0.024	−0.149*
Hip (cm)	−0.057	0.030	−0.118*
Waist:hip ratio	−0.148**	0.012	−0.104
Glucose (mmol/L)	−0.096*	−0.018	−0.107
Insulin (mIU/L)	−0.042	0.045	−0.093
GGT (U/L)	−0.068	−0.065	−0.045
AST (U/L)	0.082	−0.053	0.166**
ALT (U/L)	0.083	0.019	0.165**
Cholesterol (mmol/L)	0.048	−0.080	0.046
TG (mmol/L)	−0.229***	−0.115	−0.311***
LDL (mmol/L)	0.056	−0.003	0.052
HDL (mmol/L)	0.160***	−0.050	0.191**
CRP (mg/L)	−0.081	−0.048	−0.117*
Total SFA	−0.031	−0.083	0.000
Total MUFA	−0.162***	−0.165*	−0.195**
Total n-6PUFA	−0.652***	−0.602***	−0.674***
Total n-3PUFA	0.971***	0.927***	0.970***
Fatty Liver Index	−0.165***	−0.013	−0.206***

Participants were defined as having NAFLD if they had a FLI score ≥60 [21]. FLI score includes BMI, waist circumference, TG and GGT [21].
* Statistically significant at $p < 0.05$; ** Statistically significant at $p < 0.01$; *** Statistically significant at $p < 0.001$.
[a] Values are correlation coefficients (r) calculated using Pearson product-moment correlation analyses.
[b] O3I: Omega-3 index = erythrocyte EPA% + DPA% of total erythrocyte fatty acids [50].

Table 5
The prevalence of NAFLD in the lowest and highest quartiles of omega-3 index scores, and the prevalence of NAFLD within the male and female subgroups for those quartiles.[a]

O3I[b] quartiles	All participants			Males			Females		
	Number of people without NAFLD (n = 259)	Number of people with NAFLD (n = 216)	OR (95% CI)	Number of people without NAFLD (n = 78)	Number of people with NAFLD (n = 110)	OR (95% CI)	Number of people without NAFLD (n = 181)	Number of people with NAFLD (n = 106)	OR (95% CI)
1	60 (50.4%)	59 (49.6%)	1.00 (reference)	25 (43.1%)	33 (56.9%)	1.00 (reference)	35 (57.4%)	26 (42.6%)	1.00 (reference)
2	53 (44.9%)	65 (55.1%)	1.25 (0.75–2.08)	20 (35.7%)	36 (64.3%)	1.36 (0.64–2.90)	33 (53.2%)	29 (46.8%)	1.18 (0.58–2.41)
3	74 (61.7%)	46 (38.3%)	0.63 (0.38–1.06)	17 (48.6%)	18 (51.4%)	0.80 (0.35–1.86)	57 (67.1%)	28 (32.9%)	0.66 (0.34–1.31)
4	72 (61.0%)	46 (39.0%)	0.65 (0.39–1.09)	16 (41.0%)	23 (59.0%)	1.09 (0.48–2.48)	56 (70.9%)	23 (29.1%)	0.55 (0.27–1.11)
P-trend			<0.05			ns			<0.05

Participants were defined as having NAFLD if they had a FLI score ≥60 [21]. FLI score includes BMI, waist circumference, TG and GGT [21]. Odds Ratio (OR) calculated using lowest quartile as reference. Trends in proportion are assessed using linear-by-linear association. ns, not statistically significant at $p < 0.05$.

[a] Quartile ranges were determined using the data from all participants and were as follows [1]: 4.16%–6.84% [2]; 6.85%–7.99% [3]; 8.00%–9.78% [4]; 9.80%–14.81%.

[b] O3I: Omega-3 index = erythrocyte EPA% + DPA% of total erythrocyte fatty acids [50].

the second block of the analyses accounted for a further 32.5% of the variation in FLI (Model 2: $R = 0.618$, $p < 0.001$; $\Delta R^2 = 0.325$, $p < 0.001$). The inclusion of O3I at the final block of the analysis also contributed significantly to the prediction of FLI ($\Delta R^2 = 0.005$, $p < 0.05$), although its individual contribution to the predictive power was small. When all seven independent variables were included in the model, with the exception of age, all contributed uniquely to the multivariate correlation (Table 6).

Table 6
Hierarchical multiple regression analyses of the relationship between FLI and Omega-3 index (O3I) in all participants (n = 475) after controlling for potentially confounders.[a]

	All participants	
Block 1 Model		
Model Statistics	$R = 0.239$, $R^2 = 0.057$, Adj. $R^2 = 0.053$, $F(2,469) = 14.145$, $p < 0.001$	
$R^2\Delta$ Statistics	$R^2\Delta = 0.057$, $F(2,469) = 14.145$, $p < 0.001$	
Variables	β	*p*
Age (yrs)	−0.119	0.008
Gender	−0.202	0.000
Block 2 Model		
Model Statistics	$R = 0.618$, $R^2 = 0.381$, Adj. $R^2 = 0.373$, $F(6,465) = 47.791$, $p < 0.001$	
$R^2\Delta$ Statistics	$R^2\Delta = 0.325$, $F(4,465) = 60.995$, $p < 0.001$	
Variables	β	*p*
Age (yrs)	−0.050	0.172
Gender	−0.098	0.015
F. Glucose (mmol/L)	0.179	0.000
Insulin (mIU/L)	0.360	0.000
HDL (mmol/L)	−0.211	0.000
CRP (mg/L)	0.128	0.001
Block 3 Model		
Model Statistics	$R = 0.622$, $R^2 = 0.387$, Adj. $R^2 = 0.377$, $F(7,464) = 41.783$, $p < 0.001$	
$R^2\Delta$ Statistics	$R^2\Delta = 0.005$, $F(1,464) = 3.927$, $p < 0.05$	
Variables	β	*p*
Age (yrs)	−0.048	0.190
Gender	−0.089	0.028
F. Glucose (mmol/L)	0.175	0.000
Insulin (mIU/L)	0.361	0.000
HDL (mmol/L)	−0.203	0.000
CRP (mg/L)	0.122	0.001
O3I	−0.074	0.048

* Statistically significant at $p < 0.05$; ** Statistically significant at $p < 0.01$; *** Statistically significant at $p < 0.001$.

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

4. Discussion

This is the first study to investigate an association between O3I and NAFLD amongst older adults (≥65 years) using an objective measure of long-term n-3PUFA status. The study found an inverse association between O3I and NAFLD in female but not male participants, suggesting that sex differences may be an important consideration when evaluating the efficacy of n-3PUFA in the prevention of NAFLD. Previous research has drawn inconsistent conclusions on correlations between n-3PUFA levels and NAFLD. A recent systematic review by Parker et al. [3] reported that n-3PUFA supplementation was effective in the reduction of hepatic steatosis in adults aged >18 years, with one study [38] reporting a reversal of hepatic steatosis to normal liver function in 33.4% of participants. Despite some design flaws (randomization and blinding) in the reviewed studies, the findings indicated a potential role for n-3PUFA in the prevention and treatment of NAFLD.

To date, the majority of intervention studies have examined the effect of n-3PUFA supplementation on NAFLD in mixed-gender participant groups [3,19], with the exception of a few studies that were conducted specifically in women and that showed a significant reduction in hepatic steatosis [39,40]. A recent study demonstrated that O3I was associated with liver fat concentration [38,39]. However, when O3I was included in a multivariate analysis, it did not significantly improve the overall capacity of demographic, anthropometric and blood markers to predict NAFLD [38,39,41]. In this study, 70% of the participants were men, which may have attenuated the correlation observed. The use of mixed gender groups may potentially explain some of the inconsistencies found in reported outcomes and indicates the need for further sex and age specific intervention studies to more clearly delineate the role of n-3PUFA in NAFLD.

The prevalence of NAFLD in the current study population of older adults aged 65 years and over was 45% compared with 3% in children [42], 5–39% in adults aged 18–39 years [5] and 40% in adults aged 50 years and older [5]. Older populations may have an increased risk of developing NAFLD, due to the increased prevalence of co-morbidities and metabolic abnormalities in this age group, as well as decreased levels of physical activity [43]. The increased prevalence of NAFLD in this population emphasizes the need for the development of strategies to prevent and treat NAFLD in elderly populations.

BMI, waist circumference, TG and GGT were also individually negatively correlated with O3I, and the prevalence of NAFLD was higher in participants with lower O3I scores.

In our study, 69% of participants with NAFLD were classified as obese (BMI ≥30.0 kg/m^2). Recent research investigating the

association between n-3PUFA levels and BMI have shown inconsistent findings. A review by Buckley and Howe, 2009 [44] reported reductions in adiposity with increasing n-3PUFA levels, however the majority of the studies discussed were short-term and conducted in conjunction with energy-restricted dietary - interventions. In contrast, a double blind randomized controlled trial (RCT) conducted in young adults aged 20–35 years reported no significant alterations in anthropometric measurements with increasing n-3PUFA supplementation [45]. In the current study, an inverse association between O3I and BMI was found in females alone. This finding further supports the need for gender specific intervention trials. Buckley and Howe discussed a number of possible mechanisms by which n-3PUFAs may affect weight/BMI [44], including n-3PUFA mediated alterations in gene transcription factors that play a role in hepatic lipid production and maintenance, in particular peroxisome proliferator-activated receptor α (PPARα) and sterol regulatory element-binding protein 1 (SREBP-1) [7]. Individuals with obesity have been shown to have a lower dietary intake of n-3PUFA. As n-3PUFA are known to regulate the expression of hepatic gene transcription factors such as PPARα and SREBP-1 [7,13] resulting in the induction of hepatic fatty acid oxidation and inhibition of fatty acid synthesis, reduced n-3PUFA levels may predispose obese individuals to NAFLD.

Previous studies have reported that high doses of n-3PUFA supplementation significantly lower serum triglycerides [18], another component of the FLI. These findings are supported by more recent evidence, with a 2011 RCT conducted in men and post-menopausal women, reporting a significant reduction in serum triglycerides with a higher dose (3.4 g/day) of n-3PUFA supplementation [46]. In our study, however, females alone showed an inverse association between O3I and TG.

Similar to a recent animal study investigating the effects of n-3PUFA and n-6PUFA on GGT [47], we found no association between O3I and GGT. In the former study, Ketsa and Marchenko found that GGT and ALT levels were not affected by n-3PUFA supplementation, but reported a reduction in AST [47]. In the present study, in females, both AST and ALT levels were positively correlated with O3I.

The O3I values obtained in our study population are relatively high in comparison to existing O3I studies [48]. This elevation may result from the methodology used to analyse the erythrocyte membrane fatty acids, and/or the age of the study participants (65–95 years) [49].

The gold standard for diagnosis of NAFLD is liver biopsy, but it is not frequently performed due to its invasive nature and low acceptance rate. Ultrasonography is more commonly used to detect NAFLD because it is non-invasive, sensitive, low-cost and easy to perform. In the present study, we used FLI, a validated tool to predict NAFLD, however, the lack of histological findings or a direct measure of NAFLD remains a drawback of our investigation.

Strengths of this study include the relatively large sample size, and consequent generalizability to the aged population, and the use of a long-term objective biomarker of O3I that allowed a more accurate measure of long-term n-3PUFA status. Additional limitations include cross sectional design, self-reported dietary intake and lack of separation of PUFA intake into n-3PUFA and n-6PUFA. NAFLD is defined as the hepatic expression of the metabolic syndrome. Medications used to treat metabolic syndrome may therefore be considered as potential confounders to NAFLD research. In the current study, a small number of participants were taking medications for type 2 diabetes mellitus, hypertension, lowering lipids and weight loss. These medications may have altered NAFLD expression by influencing one or more of the four variables incorporated into the calculation of FLI.

5. Conclusion

In conclusion, we found a significant inverse association between erythrocyte O3I and FLI scores in older adults, supporting a relationship between O3I and NAFLD. The association between O3I and FLI was gender-specific, being only apparent in females. Sex-based RCT's using objective measures of NAFLD should be conducted to validate and further investigate these findings.

Author contributions

MLG MV and ML designed research; SN KK and MR conducted research; MR analysed data; MR, MLG, SB, KK and SN wrote the paper; MR had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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 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 are also grateful to Ms Melissa Fry for her assistance in the fatty acid analysis.

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