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Abstract
Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) are beneficial for health. To date there is no specific food frequency questionnaire (FFQ) to assess LC n-3 PUFA intakes. The objective of this study is to validate our newly developed FFQ by comparison with LC n-3 PUFA content of both red blood cells (RBC) and plasma, expressed as a percentage of total fatty acids. Fifty-three healthy male and female subjects were recruited from Wollongong, Australia. Average LC n-3 PUFA intakes (mg/day) were estimated using the new FFQ. RBC and plasma fatty acids were assessed using gas chromatography. Spearman correlation co-efficients assessed the linear relationship between FFQ intakes and both RBC and plasma fatty acids.) The results show that there were significant Spearman's correlation co-efficients between the FFQ intakes and RBC (and plasma) fatty acids for total LC n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (0.50 (0.54), 0.39 (0.54) and 0.40 (0.48) respectively) but not for docosapentaenoic acid (DPA). The FFQ was also an effective ranking tool. In summary, the FFQ is a valid method based on erythrocyte and plasma fatty acids as biochemical markers. In conclusion, the new FFQ is a valid method that can be used to estimate the LC n-3 PUFA intake of adults.

Keywords
Omega-3 polyunsaturated fatty acids, eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, red blood cell, plasma, food frequency questionnaire, intakes, validation, biomarker

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TITLE PAGE

Biomarker Validation of a Long-Chain Omega-3 Polyunsaturated Fatty Acid Food Frequency Questionnaire

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KEYWORDS: Omega-3 polyunsaturated fatty acids, eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, red blood cell, plasma, food frequency questionnaire, intakes, validation, biomarker.

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FOOTNOTES PAGE

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Abbreviations Used:

DHA - docosahexaenoic acid; DPA – docosapentaenoic acid; EPA - eicosapentaenoic acid; FFQ - food frequency questionnaire; FR – food record; ISSFAL – International Society for the Study of Fatty Acids and Lipids; LC n-3 PUFA - long chain omega-3 polyunsaturated fatty acids; NNS - National Nutrition Survey; RBC - red blood cells.

ABSTRACT

Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) are beneficial for health. To date there is no specific food frequency questionnaire (FFQ) to assess LC n-3 PUFA intakes. The objective of this study is to validate our newly developed FFQ by comparison with LC n-3 PUFA content of both red blood cells (RBC) and plasma, expressed as a percentage of total fatty acids. Fifty-three healthy male and female subjects were recruited from Wollongong, Australia. Average LC n-3 PUFA intakes (mg/day) were estimated using the new FFQ. RBC and plasma fatty acids were assessed using gas chromatography. Spearman correlation co-efficients assessed the linear relationship between FFQ intakes and both RBC and plasma fatty acids.) The results show that there were significant Spearman's correlation co-efficients between the FFQ intakes and RBC (and plasma) fatty acids for total LC n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (0.50 (0.54), 0.39 (0.54) and 0.40 (0.48) respectively) but not for docosapentaenoic acid (DPA). The FFQ was also an effective ranking tool. In summary, the FFQ is a valid method based on erythrocyte and plasma fatty acids as biochemical markers. In conclusion, the new FFQ is a valid method that can be used to estimate the LC n-3 PUFA intake of adults.

INTRODUCTION

The various health benefits of consuming the long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been reported widely (1-6). The LC n-3 PUFA are obtained predominately from fish, seafood, meat and eggs (7, 8) and in recent years from enriched food products such as bread, milk, margarine and eggs. Recommendations for dietary intakes of LC n-3 PUFA vary considerably from the consumption of two fish meals a week (9) to EPA plus DHA intakes of 500mg/day (10) and the Japanese recommend consumption of LC n-3 PUFA of 1.6g/day (11).

Currently there is no tailor made food frequency questionnaire (FFQ) to assess LC n-3 PUFA intakes. We have developed a new FFQ that estimates the dietary intakes of LC n-3 PUFA. The FFQ was found to be highly reproducible and valid compared to 3-day weighed food records in 53 healthy adults (12). However, as biochemical measurements of specific nutrients in the blood or other tissues can also provide useful assessments of nutrient intake (13), it is desirable to validate the FFQ further using biomarkers of LC n-3 PUFA intake.

There are several choices of a biomarker for the measurement of LC n-3 PUFA including fatty acids in adipose tissue (14), red blood cells (RBCs), platelets, plasma, cholesterol esters, phospholipids (13) and potentially cheek cells (15). RBCs may be a useful marker as they can provide an indication of the previous 120 days intake of LC n-3 PUFA, the approximate lifespan of a RBC (16). RBCs were found to be a good biomarker of LC n-3 PUFA intake as EPA and DHA were incorporated into RBCs according to dose (17). Plasma has also been found to reflect LC n-3 PUFA intake in various studies (18, 19).

The aims of this study were to 1) compare estimates of dietary LC n-3 PUFA intakes using the new FFQ to previously published results; 2) compare estimates of dietary LC n-3 PUFA intakes using the new FFQ to RBC fatty acids; 3) compare estimates of dietary LC n-3 PUFA intakes to plasma fatty acids, 4) determine the efficacy of the FFQ to rank individuals into quintiles of intakes of LC n-3 PUFA by comparison to quintiles of RBC and plasma fatty acids.

MATERIALS AND METHODS

Subject Recruitment and Clinic Visits

Poster and e-mail advertisements were used to recruit staff and students from the University of Wollongong, Australia. There were no exclusion criteria with the only requirement being that subjects had maintained a constant diet over the previous three months. Vegetarians were encouraged to volunteer to ensure a wide range of dietary habits among the subjects. Based on RBC data from a previously unpublished study, it was determined that a minimum of 50 subjects should be sufficient to detect an effect size of 1 with more than 99% power at a significance level of 0.05, assuming that the correlation between methods is 0.6. The effect size is the difference between the means of the two methods, divided by the standard deviation (SD) of either method.

Subjects attended a single clinic appointment and came in after an overnight fast to provide a fasted blood sample for RBC and plasma fatty acid analysis. Digital scales and a wall-mounted stadiometer were used to measure weights and heights, respectively. Subjects then completed the FFQ. Approval to conduct the study was obtained from the University of Wollongong Human Research Ethics Committee and subjects gave their written consent.

LC n-3 PUFA Food Frequency Questionnaire and Determination of Intakes

The 28 item semi-quantitative FFQ consists of a list of food items that are currently the only major contributors to LC n-3 PUFA intake, and is available from BM. The foods included in the 28 questions are from the following food groups: fish and seafood (6 questions), meats (12), cereals (4), fat spreads (2), dairy (2), eggs (2) including LC n-3 PUFA fortified products such as breads, milks, margarines and eggs. Fish oil capsules are also included in the questionnaire. The FFQ also asks the subject to estimate ususal meat serve size in terms of pictures of meat on a plate. The dietary validation of this FFQ has been published

elsewhere (12). The FFQ was self-administered by subjects and took approximately 15 minutes to complete. The LC n-3 PUFA intakes of FFQ and the 3 day weighed Food Record (FR) were determined as described by Sullivan *et al.* (12).

Comparison to other published intakes

To determine if the FFQ is measuring a plausible intake, another dietary intake comparator was necessary. Therefore, the intakes estimated using the FFQ were compared with those of a larger Australian sample using a 24 hour recall method taken from the 1995 National Nutrition Survey (NNS) to estimate LC n-3 PUFA intake (20). The NNS collected data from 10,591 Australians aged 19 years and older, but at a time when omega-3 fortified foods were not on the market. The Wilcoxon signed rank test was used to compare the FFQ data (without the inclusion of fortified foods or fish oil capsules) to the 3 day weighed food record (FR) (as previously described (12)) with comparison to the NNS data (21).

Blood collection, preparation and analysis of RBCs and plasma fatty acids

Blood was collected into tubes containing ethylenediamine tetra-acetic acid (EDTA) and placed on ice. The samples were separated by centrifugation (10 min, 3000rpm, 4°C). The plasma was separated from the sample into labelled eppendorf tubes. The white buffy layer was removed and discarded and the remaining RBCs were transferred into labelled eppendorf tubes. RBC and plasma samples were stored at -80°C until analysed.

RBC membranes were prepared for fatty acid analysis as described by Ridges *et al.* (22). The total volume of the resuspended pellets was transferred into glass tubes for direct transesterification as described by Lepage and Roy (23). Briefly, 2ml of methanol: toluene (4:1) was added to each sample. While vortexing, 200µl of acetyl chloride was added drop wise to each sample using a positive displacement pipette. Samples were then heated for 60

minutes at 100°C in a heating block. After the tubes had been cooled in cold water, 3ml of potassium chloride (10%) and 100µl of toluene were added to each tube before centrifugation for 10 minutes (3000rpm, 4°C). The fatty acid methyl esters, contained in the upper toluene phase, were removed and placed into GC vials. The fatty acid methyl esters were analysed by flame-ionisation gas chromatography (model GC-17A, Shimadzu) using a 30m x 0.25mm internal diameter capillary column. Individual fatty acids were identified upon comparison with known fatty acid standards (Supelco F.A.M.E. mix C4-C24 (plus added DPA), #18919-1AMP, Sigma, Australia).

Total plasma fatty acids were determined using the method by Lepage and Roy (23). Aliquots of 200µl of plasma were transferred into glass tubes for direct transesterification (23) and as briefly described above, however instead of using 3ml potassium chloride (10%), 5ml potassium chloride (5%) was used and instead of adding 100µl toluene, no toluene was added. Fatty acid methyl esters were analysed as described above.

Comparison of FFQ and LCn-3 PUFA content of RBCs and plasma

Means (SD) were calculated for the total LCn-3 PUFA, EPA, docosapentaenoic acid (DPA) and DHA content (as a percentage of total fatty acids) of the RBCs and plasma. Spearman's correlation coefficients were determined for the relationship between FFQ estimated daily intakes and LCn-3 PUFA content of both RBCs and plasma.

Quintile agreement

Subjects were ranked in ascending order of their daily total intake of LC n-3 PUFA estimated from the FFQ and both RBC and plasma fatty acids. They were then separated into quintiles. Quintiles were determined by assessing the actual intakes from 53 subjects ranging from 0 to 1100mg/day and then dividing the subjects into 5 equal groups, where

quintile 1 equals 0-98mg/d, quintile 2 equals 99-188mg/d, quintile 3 equals 189-304mg/d, quintile 4 equals 305-449mg/d and quintile 5 equals 450-1100mg/d. The percentage agreement was determined between the quintile assignments using the FFQ and RBC fatty acids and separately for the FFQ and plasma fatty acids.

Statistical Analysis

Means (SD) were calculated for the total LCn-3 PUFA, EPA, DPA and DHA content (as a percentage of total fatty acids) of the RBCs and plasma. The relationships between FFQ estimated daily intakes and LCn-3 PUFA content of both RBCs and plasma were assessed by Spearman's correlation coefficients.

Quintile agreement was assessed by determining the percentage agreement between quintile assignments using the FFQ and RBC fatty acids and for FFQ and plasma fatty acids.

Statistical analysis was performed using JMP statistical analysis program (Version 5.1, SAS Institute, Cary, NC, USA). Statistical significance was set at $\alpha = 0.05$ for all analyses.

RESULTS

Fifty-three subjects (20 male and 33 female) were recruited for the validity study, including seven self-reported vegetarians. Two vegetarians were vegans, one ate extremely small amounts of fish and two individuals were lactoovovegetarians. The remaining two reported eating significant amounts of fish in the FFQ. The subject characteristics are presented in Table 1.

Comparison of our FFQ LC n-3 PUFA intakes to the National Nutrition Survey (NNS) of Australia

Table 2 shows the LC n-3 PUFA intakes data estimated from the FFQ (excluding fortified foods and fish oil capsules) in comparison to the analysis of the National Nutrition Survey (NNS) of Australia conducted by Howe *et al.* (20). There were no significant differences between the estimates in this study using the FFQ and the NNS data.

Comparison of our LC n-3 PUFA FFQ to the Fatty Acid Content of RBCs and plasma

Comparison of the LC n-3 PUFA intakes estimated from the FFQ to the percentage of the total fatty acid content of RBCs and plasma are shown in Table 3.

Bivariate plots of the FFQ estimation of LC n-3 PUFA intakes and percentage of total fatty acid content of the RBCs showed that there were good, significant relationships for total LC n-3 PUFAs, EPA, and DHA (Figures 1a, 1b and 1d). There was no apparent relationship for DPA (Figure 1c). Similar patterns were observed for the relationship between the FFQ estimated intakes and LC n-3 PUFA content of plasma. As seen with the RBCs, there was no relationship between FFQ estimation of DPA intakes and the content of plasma as indicated by the non-significant Spearman's correlation of 0.09.

The efficacy of the FFQ to rank individuals into quintiles of intakes of LC n-3 PUFA

Subjects were also assigned quintiles based on total LC n-3 PUFA content of the RBCs (% of total fatty acids) (Table 4). Forty percent of subjects were correctly assigned into the same quintiles while 75% of subjects were assigned to either the same or adjacent quintile. The FFQ was most successful at identifying individuals with very low intakes. Similar results were obtained for agreement of quintile assignment between FFQ total LC n-3 PUFA intakes and total LC n-3 PUFA content of plasma (Table 4). Five out of the seven vegetarian subjects were found in the first quintile for both analyses.

DISCUSSION

Our new FFQ estimates the dietary intakes of LC n-3 PUFA very well as the results compare favourably to the results from the NNS of Australia (Table 2, and ref. 20). The NNS intakes of EPA and DPA were slightly higher and the NNS intakes of DHA were slightly lower than our study, which could be explained by the fact that our study group contained 7 vegetarians. With meat being a rich source of DPA (24), the inclusion of 7 vegetarian subjects (who did not eat meat) in this study may account for the lower DPA intakes.

Other studies have used various biomarkers to validate newly developed or existing FFQs, although not many have been validated for LC n-3 PUFA intake and those that do use biomarkers other than RBCs. Woods *et al.* (25) validated an existing semi-quantitative FFQ for measuring fish intake with plasma fatty acid composition. That FFQ was assessing overall dietary intake over the preceding 12 months and was originally developed for use by the Anti Cancer Council of Victoria. Significant Pearson correlation co-efficients were obtained between percentage of plasma fatty acids and grams of steamed, grilled or baked fish and grams of total non-fried fish consumed ($r = 0.33$ for DHA and 0.34 for total LC n-3 PUFA). Significant Pearson correlation co-efficients were also obtained between the percentage of total plasma n-3 PUFAs and the same two variables which were both found to be 0.33. Our FFQ shows higher Spearmans correlation co-efficients ($r=0.50$ for total LC n3 PUFA, $r=0.39$ for EPA and $r=0.40$ for DHA), hence our newly developed FFQ is an improvement on previously validated FFQs to estimate LC n-3 PUFA intakes (25).

There are a few reasons why our new FFQ shows higher correlations. Firstly, in Woods *et al.* (25), intakes of fish (steamed, baked, grilled, fried and takeaway fish) and tinned fish

were calculated without the inclusion of other types of seafood. There was no distinction between different fish species which are known to have large variations in LC n-3 PUFA content (24). Secondly, other foods that contain LC n-3 PUFAs were not included in the analysis such as eggs and meat (8) and these may account for nearly 50% of LC n-3 PUFA intakes (20). Finally, a major limitation was that at the time of the study there was no Australian database for the estimation of DHA and EPA intakes from the FFQ, which has now been rectified by Mann *et al.* (26).

Our correlation co-efficients between RBC and dietary intakes of LC n-3 PUFA compare favourably to the study by Anderson *et al.* (18). Correlation coefficients between plasma phospholipid fatty acids and dietary intakes of EPA and DHA were 0.51 and 0.49, respectively. Their correlation between fish intake and n-3 PUFA in plasma phospholipids was 0.37 whilst our correlation co-efficient of total intake of LC n-3 PUFA and RBC LC n-3 PUFA was 0.5. Their lower correlation between fish intake and n-3 PUFA in plasma phospholipids further highlights the fact that foods other than fish and seafood contribute to LC n-3 PUFA intake (20).

The mean percentages of LC n-3 PUFAs in RBCs in our study were comparable to those determined in other studies (17, 26). LC n-3 PUFA content of plasma was however lower than reported in other studies (25, 27). RBCs and plasma were found to be good biomarkers of total LC n-3 PUFA, EPA and DHA intakes as highlighted by the significant Spearman's correlation co-efficients but RBC and plasma were not found to be good biomarkers of DPA intakes (r=0.05). Significant correlations between dietary intakes and RBC fatty acids were expected for EPA and DHA as both fatty acids have been found to be incorporated into RBCs by dose (17). However, DPA intakes estimated from the FFQ were found to be highly reproducible with a highly significant Spearman correlation co-efficient of 0.89 (12). This

evidence strongly suggests that the lack of correlation is not due to the FFQ instrument itself and that it is more likely due to biological or metabolic factors and this warrants further investigation.

Quintile assignment proved that the FFQ was able to adequately identify individuals according to their RBC levels of LC n-3 PUFA. Five out of the seven vegetarians were correctly assigned to the first (lowest intake) quintile based on intakes measured by the FFQ and LC n-3 content of the RBCs. Agren *et al.* (28) also found that Finnish vegans had significantly lower EPA, DPA and DHA in their RBCs than omnivorous subjects. The other two self-reported vegetarians, as expected, had higher LC n-3 PUFA content of RBCs due to significant intakes of fish and seafood. Overall, the quintile assignment in this study was better than or consistent with other studies. The FFQ used by Erkkola *et al.* (29) classified 62% of individuals into the same or adjacent quintiles based on total n-3 PUFA intakes. The ability of this FFQ to classify more than 75% of individuals into identical or adjacent quintiles may be useful in clinical settings to identify individuals with low LC n-3 PUFA status.

In summary, the FFQ performed well against the RBC membrane and total plasma fatty acids, apart from the lack of correlation between DPA intakes and DPA content of both RBCs and plasma, suggesting that these may not be good biomarkers of DPA intake. The FFQ is therefore an adequate dietary assessment tool for the estimation of total LC n-3 intakes in healthy Australian adults and would be a useful screening tool for low intakes of LC n-3 PUFAs. It is an appealing alternative to other dietary assessment methods due to its validity, low subject burden and low cost and being very fast to complete. In conclusion, the FFQ is a valid dietary assessment tool for the estimation of dietary LC n-3 PUFAs.

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TABLES

Table 1. Subject characteristics (female n = 33, male n=20).

Subject Characteristic	Mean (SD)	Range
Age (years)	34.6 (11.6)	19-58
Height (cm)	168.5 (10.0)	145-187
Weight (Kg)	68.4 (13.4)	43-100
BMI (Kg/m^2) *	23.9 (3.1)	18.7-33.0

*BMI= body mass index

Table 2. Average daily intakes (mg/day) of the individual and total LC n-3 PUFAs without the inclusion of fortified foods or fish oil capsules.

Fatty acid	FFQ intakes (mg/day)*	NNS 1995 intakes (Howe et al, 2005)	Wilcoxon signed rank test
Total LCn-3 PUFAs	259 (209)	246	0.635
EPA	73 (63)	75	0.755
DPA	52 (41)	71	0.405
DHA	134 (120)	100	0.544

*expressed as mean (SD)

Table 3. Relationship between biomarker fatty acids and intakes from the FFQ (n=53).

FATTY ACID	RBC fatty acids*[a]	Plasma fatty acids*[a]	FFQ intakes*	Spearman correlation co-efficient RBC vs FFQ	Spearman correlation co-efficient Plasma vs FFQ
Total	6.57	3.35	259	0.50[b]	0.54[b]
LC n-3	(1.27)	(1.07)	(209)		
EPA	0.43	0.84	73	0.40[c]	0.54[b]
	(0.19)	(0.42)	(63)		
DPA	2.08	0.48	52	0.05	0.09
	(0.41)	(0.11)	(41)		
DHA	4.06	2.02	134	0.39[c]	0.48[b]
	(1.12)	(0.71)	(120)		

*mean (SD)
[a] expressed as % of total fatty acids
[b] significant at p< 0.001
[c] significant at p< 0.01

Table 4. Agreement of quintile assignment between FFQ total LC n-3 PUFA intakes and total LC n-3 PUFA content of both RBCs and plasma (n = 53).

Quintile	FFQ intakes vs. RBCs			FFQ intakes vs. plasma		
	Same quintile	Adjacent quintile	Incorrectly classified	Same quintile	Adjacent quintile	Incorrectly classified
1 (n= 10)	8	1	1	7	2	1
2 (n= 11)	2	5	4	2	5	4
3 (n= 11)	5	5	1	3	6	2
4 (n= 11)	3	4	4	1	8	2
5 (n= 10)	3	4	3	2	5	3
Total no. (%)	21 (40)	19 (36)	13 (24)	15 (28)	26 (49)	12 (23)

Figures

Figure 1a. The relationship between estimated total LCn-3 PUFA intake from the FFQ and the LCn-3 PUFA content of the RBCs with calculated Spearman correlation co-efficient.

r=0.50
(p<0.001)

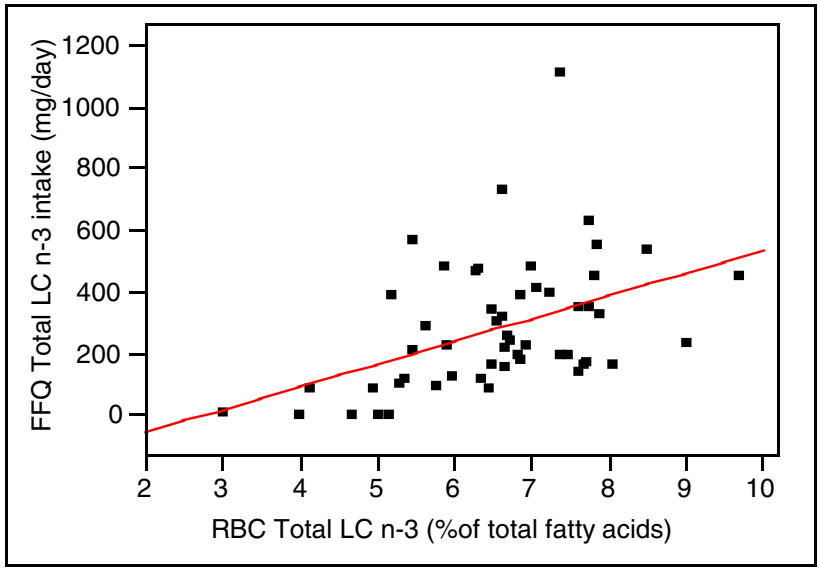


Figure 1b. Bivariate plot of EPA intakes estimated by the FFQ versus the EPA content of the RBCs as a percentage of total fatty acids with calculated Spearman correlation co-efficient.

r =0.39
(p< 0.01)

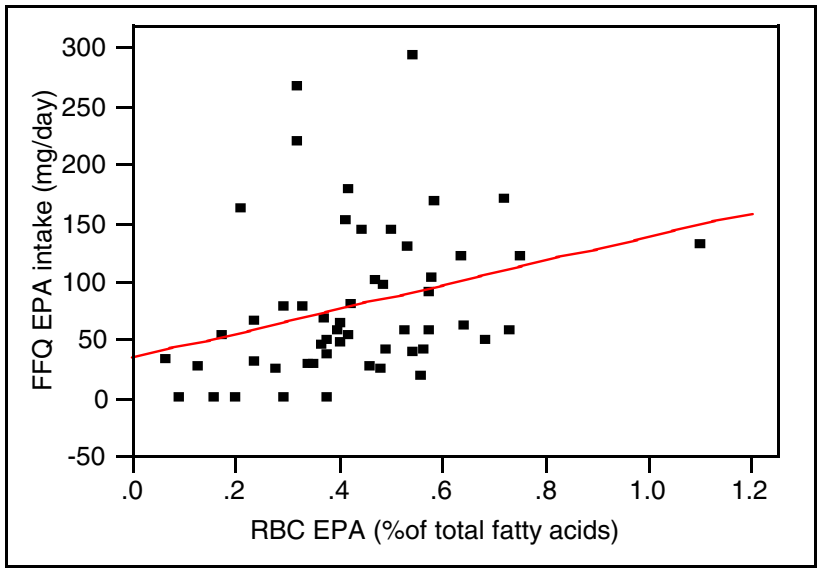


Figure 1c. Bivariate plot of DPA intakes estimated by the FFQ versus the DPA content of the RBCs as a percentage of total fatty acids with calculated Spearman correlation co-efficient. line of best fit was not fitted to this plot due to the obvious lack of relationship between the two measurements.

r = 0.05
(p = 0.71)

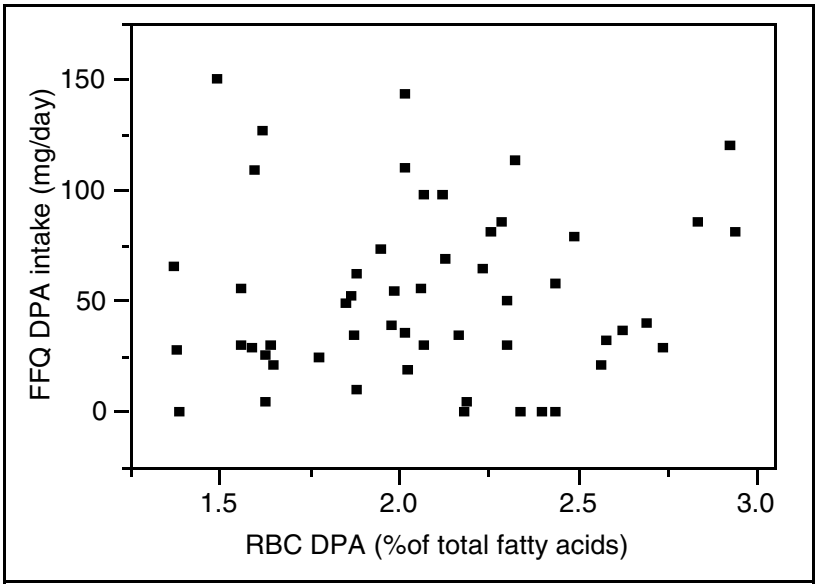


Figure 1d. Bivariate plot of DHA intakes estimated by the FFQ versus the DHA content of the RBCs as a percentage of total fatty acids with calculated Spearman correlation co-efficient.

r =0.40
(p<0.01)

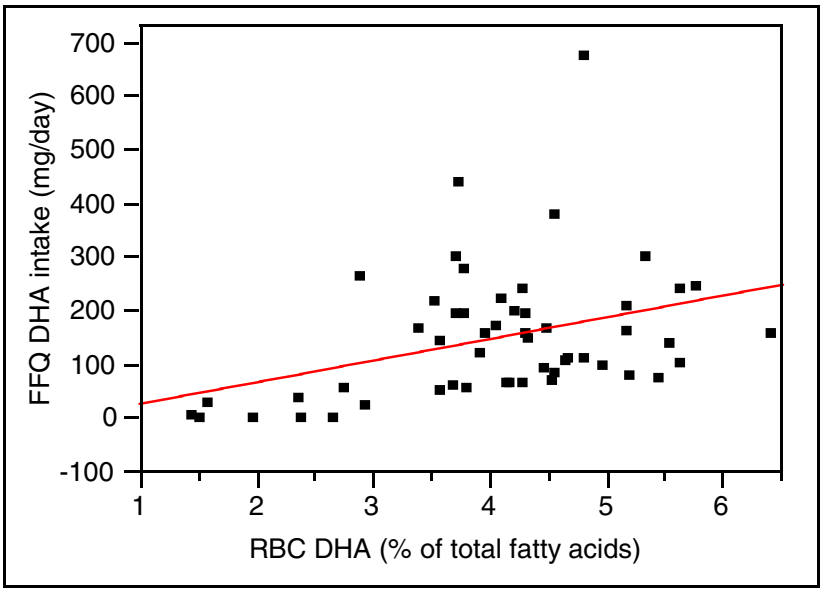
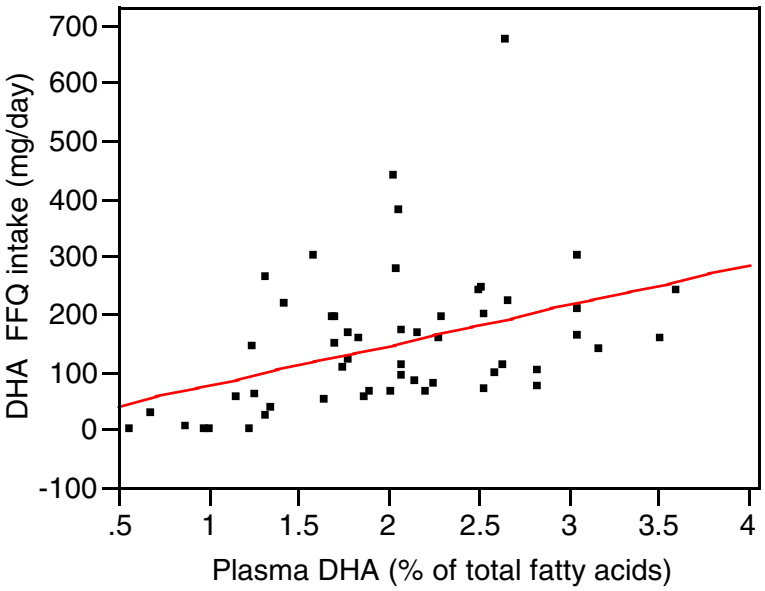
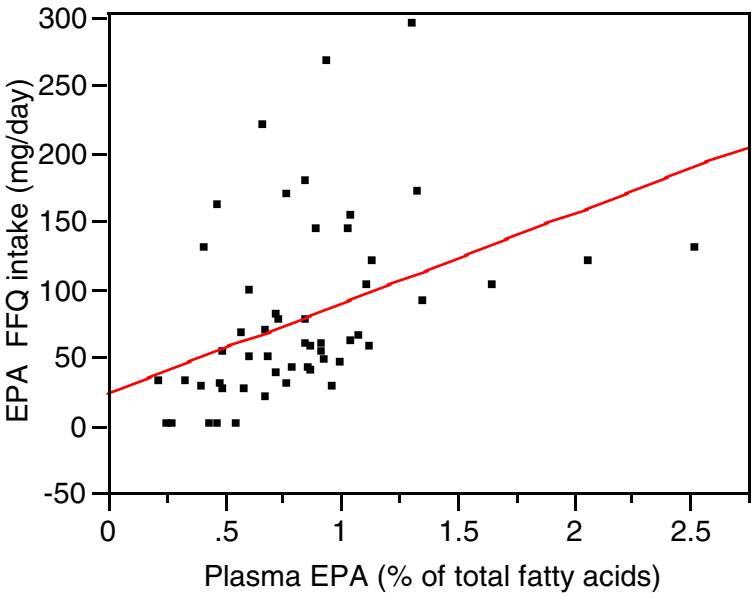


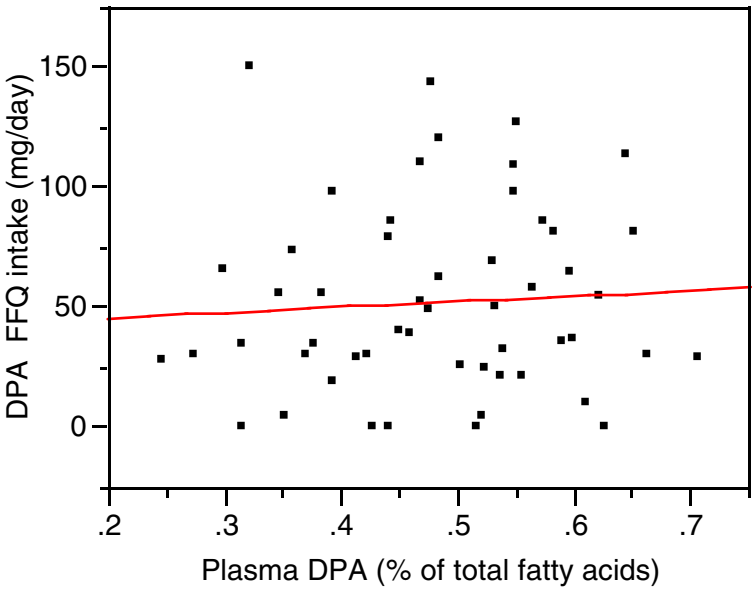
Table 3. Intake of total LC n-3 PUFAs, EPA, DPA and DHA estimated from FFQ and measured from 3-day FR in mg/day (n=45).

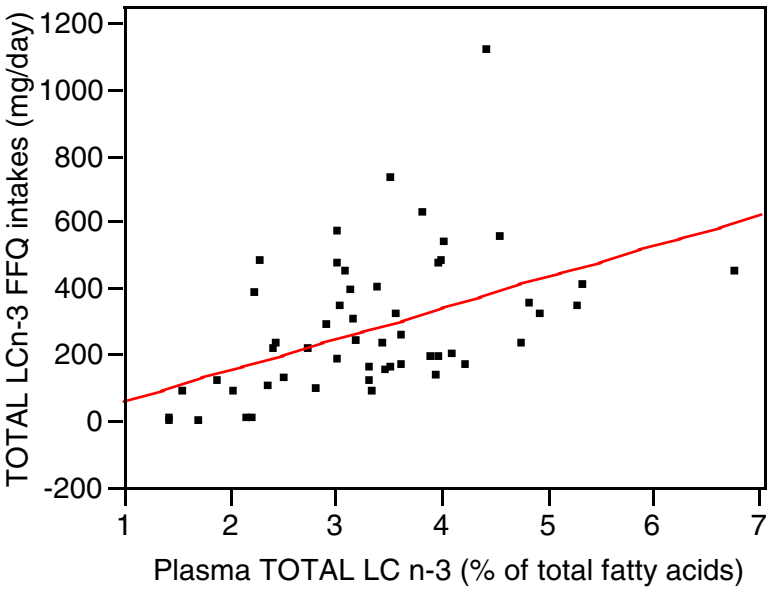
FATTY ACID	FFQ (mg/day)		FR (mg/day)		Wilcoxon-signed rank test * p =
	Mean (SD)	Median	Mean (SD)	Median	
Total LC n-3	259 (209)	212	264 (222)	234	0.822
EPA	73 (63)	58	75 (71)	65	0.670
DPA	52 (41)	48	49 (38)	40	0.487
DHA	134 (120)	108	141 (142)	125	0.391

** Significantly different at $p<0.05$


DHA FFQ intake (mg/day)
700
600
500
400
300
200
100
0
-100
.5
1
1.5
2
2.5
3
3.5
4
Plasma DHA (% of total fatty acids)


EPA FFQ intake (mg/day)
300
250
200
150
100
50
0
-50
0
.5
1
1.5
2
2.5
Plasma EPA (% of total fatty acids)


DPA FFQ intake (mg/day)
150
100
50
0
.2
.3
.4
.5
.6
.7
Plasma DPA (% of total fatty acids)


TOTAL LCn-3 FFQ intakes (mg/day)
1200
1000
800
600
400
200
0
-200
1
2
3
4
5
6
7
Plasma TOTAL LC n-3 (% of total fatty acids)