Erythrocyte levels compared with reported dietary intake of marine *n*-3 fatty acids in pregnant women

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It is well established that marine *n*-3 fatty acids measured in erythrocyte phospholipids of non-pregnant subjects reflect the subjects' intake of these fatty acids. In 135 pregnant women in the 30th week of gestation we compared intake of marine *n*-3 fatty acids and energy, estimated by a combined dietary self-administered questionnaire and interview, with fatty acids measured in erythrocyte phospholipids. Daily intake (g/d) and nutrient density of marine *n*-3 fatty acids (mg/MJ) correlated with the *n*-3 fatty acid: arachidonic acid ratio (FA-ratio) with correlation coefficients of 0·48 and 0·54 respectively. In a linear regression model with three frequency questions about marine sandwiches, marine cooked meals and fish oil as explanatory variables, and the FA-ratio as dependent variable, the multiple correlation coefficient was 0·46. Conclusions from the study were (1) levels of erythrocyte fatty acids in pregnant women may be employed as a qualitative method to rank subjects according to intake of marine *n*-3 fatty acids; (2) with respect to the power to explain FA-ratio variability, three simple marine food frequency questions were comparable with intake of marine *n*-3 fatty acids assessed by an elaborate semiquantitative dietary method involving an interview.

Dietary methods: Erythrocytes: Marine *n*-3 fatty acids: Pregnancy

Using biological markers for dietary intake has the advantage of being free of some of the constraints inherent in most methods to assess dietary intake, primarily the heavy dependence on the individuals' ability and willingness to report their intake precisely. In the case of marine *n*-3 fatty acids, several observational and supplementation studies (e.g. von Shacky *et al.* 1985; Popp-Snijders *et al.* 1986; Iso *et al.* 1989) document that levels measured in body phospholipids reflect dietary intake of these fatty acids. Thus, in a recent well-controlled experimental study a correlation coefficient of 0·9 was found between eicosapentaenoic acid measured in erythrocyte membranes and the amount of eicosapentaenoic acid ingested over a 6-week period before the blood sampling (Brown *et al.* 1990).

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These experiences from non-pregnant subjects, however, cannot be freely generalized to pregnant women, because the growing fetus is likely to be a drain on the mother's stores of essential fatty acids. The need for studies that evaluate, specifically in pregnant women, dietary methods to estimate the marine *n*-3 fatty acid intake is emphasized by current hypotheses that marine *n*-3 fatty acids ingested in pregnancy may influence pregnancy duration (Olsen *et al.* 1992), pre-eclampsia risk (Secher & Olsen, 1990), fetal growth rate (Olsen *et al.* 1990), and fetal neurodevelopment (Neuringer *et al.* 1988). We have compared intake of marine *n*-3 fatty acids, quantified by a combined self-administered questionnaire and interview, with marine *n*-3 fatty acids measured in erythrocytes of pregnant women.

MATERIALS AND METHODS

Study design

The present study was part of a larger study undertaken to investigate the suggested (Olsen *et al.* 1986) relationship between marine *n*-3 fatty acids ingested during pregnancy, and birth weight. The study ran from 15 April 1988 to 15 January 1989, interrupted by a 3-week period in August 1988. Eligible women were those scheduled to attend the routine 30th week antenatal visit at a midwifery practice that covers a geographically well-defined area of the city of Århus in Denmark. A self-administered questionnaire was mailed to the women 1 week before the scheduled visit. Provided consent was given, a supplementary 15 min structured interview was undertaken after the midwife's visit, and a blood sample was taken, processed and stored. Among eligible women, 80% (965 out of 1212) were enrolled. Among women enrolled, a random sample of 14% had their erythrocytes analysed for fatty acids; these women constitute the basis for the present study. Mean prepregnant weight of the women was 60·4 (SD 11·0) kg, and 17% were younger than 25 years and 13% were older than 34 years. The protocol was approved by the Regional Scientific-Ethical Committee of the county of Århus, Denmark.

Registering dietary intakes

The typical Danish food pattern is a cereal-based breakfast, a cold lunch meal usually consisting of open sandwiches, and a cooked evening meal. The mailed self-administered questionnaire focused on food items judged easy to quantify for the participants, and covered mainly the breakfast and lunch meals. The 15 min structured interview focused on quantifying the intake of main ingredients of cooked meals and on completing selected frequency information obtained in the questionnaire with quantitative estimates. Photographs modelling various portion sizes were used as an aid in the interviews, which were all done by a specially instructed person who also checked and corrected the completed questionnaires for possible misunderstandings. The women were asked to let the reported intake represent the latest 3 months, corresponding roughly to the second trimester of pregnancy.

Main emphasis was laid on making it possible to quantify the intake of marine *n*-3 fatty acids. When the subjects had reported intake of open sandwiches with marine foods (which is by far the most usual way of eating marine foods in cold meals in Denmark) they were asked to specify the percentage intake from a list of marine foods, and portion sizes of the three most frequent items were quantified in the interview; minor contributions of similar food types (e.g. shellfish) were in some cases aggregated. Quantification of cooked marine meals was done separately for dishes with lean (e.g. cod, plaice), medium fat (e.g. salmon, trout), fat (e.g. herring, mackerel), and minced fish.

Emphasis was also laid on energy-containing ingredients. For fat-containing items eaten with open sandwiches the subjects were asked to specify the type of the item based on a

division according to fat content (e.g. lean or fat meat), and in the interview the meat intake from cooked foods was classified into four groups according to fat content. Intakes of fat spreads and of breads were also quantified in the interview. Cereals and liquid dairy products were quantified in the mailed questionnaire. For some food types standard portion sizes (e.g. open sandwiches with meat) and standard recipes (e.g. first courses, desserts, cakes) were assumed.

Calculation of nutrient intakes

The choice of tables for food content of *n*-3 fatty acid was difficult because the tables available differ greatly in their estimates. This is due to variations in catch location and in the time of year for the catch, as well as sampling variation. Inconsistencies due to different analytical techniques may also play a role, however. We therefore chose for reference a list produced by a single laboratory that covered most of the marine food items registered in the present study, although the analyses were based on items obtained in Norwegian food stores (J. W. Andresen and G. Lambertsen, Fiskeridirektoratets Ernæringsinstitut, Bergen 1983; personal communication). The estimated contents of the various food items used to quantify total intake of marine *n*-3 fatty acids are given in Table 1. The contribution of long-chain *n*-3 fatty acids (i.e. C20 or longer) from sources other than marine foods was considered negligible.

Total individual energy intake was calculated on the basis of a computerized food table compiled for Danish foods (Levnedsmiddelstyrelsen, 1989). Most values given for food and nutrient intake in the present paper are nutrient densities, i.e. consumed amounts/d divided by energy intake/d. Adjusting for energy by including energy as explanatory variable in a regression on estimated daily intake (Willett, 1990) did not alter the estimates of association substantially.

Biochemical methods

The blood sample was immediately put on ice, and within 1 h erythrocytes were isolated as described by Dodge & Phillips (1967), rinsed and finally stored in 1·5 ml saline (9 g NaCl/l) with 0·05 g butylated hydroxytoluene (BHT)/l at −20°. The thawed erythrocytes were resuspended and a 1 ml portion was taken for analysis. Then 2 ml ice-cold methanol containing 0·1 g BHT/l was added, and the lipid extraction was carried out as described by Dodge & Phillips (1967). Methyl ester preparation and gas chromatography were performed essentially as described by Hansen & Jensen (1983) using transesterification with HCl in methanol and separating the methyl esters on a 10% SP-2330 column. The fatty acid methyl esters in total lipids were tentatively identified by comparison of retention times with those of authentic standards (Nu-Check Prep, Inc., Elysian, MN, USA) or by calculating equivalent chain lengths of homologous series of fatty acid methyl esters. For retention times where critical pairs of methyl esters were suspected, a supplementary gas chromatographic analysis was carried out on a limited number of samples. Peak areas corresponding to BHT, methyl heptadecanoate, dimethylacetals of the aldehydes 16:0, 18:0 and 18:1, or with retention times beyond 22:6, were excluded from the calculation of the peak area percentages.

The present paper focuses on comparing the estimated intake with the ratio of the sum of the three long-chain *n*-3 fatty acids, eicosapentaenoic, docosapentaenoic and docosahexaenoic acids, to arachidonic acid (the FA-ratio), which we have earlier (Olsen *et al.* 1991) used as a biochemical proxy for the intake of marine *n*-3 fatty acids. The rationale for this is that fish-oil supplementation, besides increasing the level of long-chain *n*-3 fatty acids, reduces the level of arachidonic acid in erythrocytes (e.g. von Shacky *et al.* 1985; Popp-Snijders *et al.* 1986).

Table 1. *Values used to calculate the contribution of the registered marine food items to the total individual intake of marine* n-*3 fatty acids*

	Estimated content of *n*-3 fatty acids (g/kg wet weight)
Cold food items	
Mackerel	40
Cod roe	6
Tuna	3
Breaded plaice	2*
Herring (including sardines)	20
Minced fish	0·5*
Shellfish (including shrimp, mussels and crab)	4
Salmon	12
Cod liver	100
Cooked food items	
Plaice was held representative of 'Lean fish'	4
Trout was held representative of 'Medium fat fish'	15
Herring was held representative of 'Fat fish'	20
Minced fish	0·5*
Seafood starters	1*

* Values adjusted according to recipes.

Erythrocytes for fatty acid analyses were stored for no longer than 6 months. Within this period there was no sign of degradation of fatty acids. Others have reported good stability of fatty acids in human erythrocytes during long-term storage (e.g. Bjerve *et al.* 1987; Stanford *et al.* 1991).

Statistical methods

Associations between questionnaire and biochemical variables were examined with Pearson's correlation analyses and by cross-tabulation after categorizing the variables into quintiles. Multiple linear regression analyses were undertaken in order to examine the explanatory power of the various marine factors from the questionnaire in determining the level of *n*-3 fatty acids in erythrocyte phospholipids. Adjusted coefficients of multiple determination were calculated as described by Neter *et al.* (1990).

RESULTS

Mean intake of energy was 9·7 (SD 1·99) MJ/d, of which approximately 33, 49 and 17% derived from fat, carbohydrate and protein respectively. Mean intakes of fish and long-chain *n*-3 fatty acids were 25 g/d and 0·23 g/d respectively.

Nutrient density of marine *n*-3 fatty acids (mg/MJ) tended to exhibit better correlations with the marine fatty acids quantified in erythrocyte phospholipids than did estimated daily intake (g/d; Table 2). Each of the three long-chain *n*-3 fatty acids correlated positively with nutrient density and the daily intake, whereas arachidonic acid exhibited a negative correlation. None of the C18 fatty acids correlated with nutrient density or intake of marine *n*-3 fatty acids. Nutrient density correlated with the FA-ratio with a correlation coefficient of 0·54, whereas the correlation coefficient between nutrient density and the sum of the long-chain *n*-3 fatty acids was slightly lower, 0·48.

A comparison of the dietary and the biochemical methods with respect to how they

Table 2. *Correlation coefficients* between daily intake of marine* n-*3 fatty acids relative to energy intake, and fatty acids in erythrocyte phospholipids (g/100 g) from pregnant women (n 135)*†

		Intake of marine *n*-3 fatty acids	
		Daily intake (g/d)	Nutrient density (mg/MJ)
Fatty acids in erythrocyte phospholipids (g/100 g)			
Oleic acid	18:1*n*-9	−0·15	−0·17
Linoleic acid	18:2*n*-6	0·01	0·01
Linolenic acid	18:3*n*-3	0·02	−0·01
Arachidonic acid‡	20:4*n*-6	−0·22	−0·21
Eicosapentaenoic acid	20:5*n*-3	0·55	0·58
Docosapentaenoic acid	22:5*n*-3	0·28	0·30
Docosahexaenoic acid	22:6*n*-3	0·37	0·40
Sum of long-chain *n*-3 fatty acids		0·45	0·48
Long-chain *n*-3 fatty acid:arachidonic acid ratio‡		0·51	0·54

* Pearson's product moment correlation coefficients.
† For details of procedures, see pp. 388–390.
‡ Arachidonic acid and 22:0 could not be separated on the GLC column.

ranked the women is illustrated by categorizing the women into quintiles (Table 3). Of the twenty-seven women categorized in the lowest quintile of nutrient density, ten (37%) belonged to this category according to the biochemical method; four (15%) were categorized to the highest two quintiles. Of the fifty-four women categorized in the lowest two quintiles according to the dietary method, thirty-three (60%) were classified similarly according to the biochemical method. A similar degree of agreement and disagreement between the dietary and the questionnaire methods was found in identifying women belonging to the highest quintiles. Employing daily intake rather than nutrient density gave similar results.

In Table 4 three statistical models are given that examine the associations between the various marine dietary factors on the one hand and the FA-ratio on the other. The models differ in respect to the extent to which they make use of the dietary information. Model 1 takes the simplest approach, using only three frequency questions about marine open sandwiches, marine cooked meals, and fish oil (yes/no). Model 2 employs the quantified consumption, relative to energy intake, of each marine food item. Model 3 goes one step further and combines these quantities into a single estimate of individual *n*-3 fatty acid consumption (mg/MJ), making use of information, given in Table 1, on *n*-3 fatty acid content of the registered food items.

The multiple correlation coefficient of model 1 was 0·46, and all three variables correlated significantly, and independently of each other, with the FA-ratio. The multiple correlation coefficient of model 2 was 0·64; only the consumed quantities of mackerel, cod liver, cooked lean fish, cooked medium-fat fish, and intake of *n*-3 fatty acids from fish oil correlated significantly and independently of the other explanatory variables, with the FA-ratio. The nutrient density of marine *n*-3 fatty acids (mg/MJ) and the FA-ratio correlated with the earlier mentioned correlation coefficient of 0·54, which was highly significant. The three correlation coefficients cannot be directly compared since they differ in respect to a number of determinants. Adjusted coefficients of multiple determination (Neter *et al.* 1990) were 0·44, 0·58 and 0·54, for models 1, 2, and 3 respectively.

Table 3. *A cross-tabulation of pregnant women into quintiles of estimated nutrient density of marine* n-*3 fatty acids (mg/MJ) and quintiles of the long-chain* n-*3 fatty acid:arachidonic acid ratio measured in erythrocyte phospholipids*

		Long-chain *n*-3 fatty acid:arachidonic acid ratio measured in erythrocytes					
			Distribution				
Estimated nutrient density of marine *n*-3 fatty acids (mg/MJ)			1st quintile	2nd quintile	3rd quintile	4th quintile	5th quintile	All
Distribution	Mean (SEM)	Mean (SEM)...	0·66 (0·018)	0·70 (0·021)	0·70 (0·020)	0·75 (0·018)	0·83 (0·026)	0·73 (0·0094)
1st quintile	9·4 (2·0)		10	9	4	2	2	27
2nd quintile	14 (2·5)		8	6	4	5	4	27
3rd quintile	24 (4·0)		6	7	5	5	4	27
4th quintile	33 (4·5)		2	4	9	7	5	27
5th quintile	42 (7·2)		1	2	4	8	12	27
All	24 (2·0)		27	28	26	27	27	135

Table 4. *Linear regression analyses of the long-chain* n-*3 fatty acid:arachidonic acid ratio measured in erythrocytes (*n *135), on various dietary factors as explanatory variables*

	Regression coefficient†		Simple CC‡	Part CC§
Model 1. Three simple frequency questions as explanatory variables (multiple CC:* 0·47)				
Explanatory variables				
Open sandwiches with marine foods/week	0·011	$P = 0·01$	0·17	0·19
Cooked marine food meals/month	0·015	$P = 0·0007$	0·27	0·27
Fish oil (0 = no, 1 = yes)	0·20	$P \leqslant 0·00005$	0·33	0·34
Model 2. Estimated consumed quantities (mg/MJ) of the marine foods registered in the questionnaire as explanatory variables (multiple CC:* 0·64)				
Explanatory variables				
Cold food items (ranked according to contribution to total fish consumption)				
Mackerel	84	$P \leqslant 0·00005$	0·35	0·31
Cod roe	−12	$P = 0·3$	0·01	−0·07
Tuna	27	$P = 0·2$	0·17	0·09
Breaded plaice	−12	$P = 0·5$	−0·05	−0·05
Herring (including sardines)	13	$P = 0·7$	0·21	0·03
Minced fish	−8	$P = 0·8$	−0·07	−0·01
Shellfish (including shrimp, mussels, crab)	−48	$P = 0·2$	−0·04	−0·09
Salmon	−23	$P = 0·8$	0·00	−0·02
Cod liver	393	$P = 0·04$	0·16	0·15
Cooked food items				
Lean fish	20	$P = 0·05$	0·13	0·13
Medium-fat fish	117	$P = 0·0001$	0·29	0·28
Fat fish	26	$P = 0·2$	0·23	0·09
Minced fish	1	$P = 0·9$	−0·06	0.00
Seafood starters	27	$P = 0·08$	0·07	0·12
Intake of *n*-3 fatty acids from fish oil	2·3	$P = 0·0008$	0·31	0·24
Model 3. Estimated intake of marine *n*-3 fatty acids (mg/MJ) as explanatory variable (simple CC: 0·54)				
Explanatory variable				
Marine *n*-3 fatty acids (mg/MJ)	2·6 (95% CI 1·9; 3·3)	$P \leqslant 0·00005$		

CC, correlation coefficient; CI, confidence interval.

* Square root of coefficient of determination (a measure of the explanatory power of the independent variables).

† Regression estimate of the change in fatty acid ratio per unit change in questionnaire variable.

‡ Correlation coefficient from the bivariate correlation analyses between that particular variable and the fatty acid ratio.

§ Square root of (multiple CC^2 from a regression with all variables − multiple CC^2 from regression with all variables except that particular one).

DISCUSSION

The present study documents that the FA-ratio, measured in erythrocyte phospholipids, reflects dietary intake of marine *n*-3 fatty acids in pregnant women. This biochemical measure may therefore be used as a qualitative method to rank pregnant women according to intake of marine *n*-3 fatty acids. It needs to be stressed, however, that the measurements were done in the 30th week of gestation in women with an uncomplicated pregnancy, and that the results cannot be freely extrapolated beyond these standardization criteria.

The mean energy intakes observed with the dietary intake method used in the present study are close to data reported earlier for Scandinavian women. Forsum *et al.* (1992) measured mean total energy expenditure with the doubly-labelled water technique and found a value of 9·6 (SD 2·8) MJ/d in gestation weeks 16–18 in twenty-two Swedish women with a mean body weight of 63·7 kg. Recent British estimates of energy requirement during

pregnancy suggest 8·1 MJ/d for women with a low degree of physical activity and a prepregnancy weight of 60 kg with an increment of 0·8 MJ/d during the last trimester (Department of Health, 1991). Thus, the selected method for collecting dietary data can be judged to be valid and to reflect total food intake; this also justifies the use of nutrient density figures.

A correlation coefficient of 0·54 is relatively high compared with findings from other observational studies correlating biochemical markers to estimated dietary intakes (Willett, 1990). However, it means that only 29% of the variability of the FA-ratio was accounted for by the variability in the estimated intake. Several factors are likely to attenuate the associations: first, the measurement errors inherent in the two methods; second, incomplete knowledge to construct appropriate models for the time–effect and dose–effect relationships between the intake of *n*-3 fatty acids and their appearances in erythrocyte phospholipids; third, body levels of *n*-3 fatty acids may be determined by factors other than the amounts consumed, e.g. interperson variation in the absorption and β-oxidation of *n*-3 fatty acids, and in the export of *n*-3 fatty acids to the fetus.

The negative correlation that we saw between estimated intake of *n*-3 fatty acids and erythrocyte arachidonic acid is compatible with the displacement of arachidonic acid from erythrocyte phospholipids seen (von Shacky *et al.* 1985; Popp-Snijders *et al.* 1986) after supplementation with *n*-3 fatty acids. We did not estimate dietary intake of arachidonic acid or other *n*-6 fatty acids. It is noteworthy that the FA-ratio tended to correlate better with the intake estimates than did the sum of the long-chain *n*-3 fatty acids itself. This suggests that the ratio rather than the sum should be used to rank persons according to intake of marine *n*-3 fatty acids.

Three simple frequency questions about marine open sandwiches, marine cooked meals and the use of fish oil, correlated with the FA-ratio with a multiple correlation coefficient of 0·46; after adjustment for multiple determination (Neter *et al.* 1990) the coefficient was 0·44. The benefit of the extra money and other resources spent on quantifying intake of marine *n*-3 fatty acids relative to energy, resulting in a correlation coefficient of 0·54, may therefore be regarded as modest. Using the three questions to rank the subjects would however require additional knowledge about how to combine the questions into one scale; in this population the three estimated regression coefficients from model 1 in Table 4 could be used as weights for calculating a score for such a scale. In Denmark, marine *n*-3 fatty acids typically derive from relatively few food sources, and these findings are therefore likely to be quite specific to this particular population.

In conclusion, in this population with a relatively low intake of marine foods it was possible to detect a comparatively strong correlation between erythrocyte levels and questionnaire-assessed intake of *n*-3 fatty acids. However, three simple food frequency questions were nearly as efficient in explaining variation in erythrocyte fatty acids as was intake estimated by a relatively elaborate method involving an interview and a self-administered questionnaire.

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