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Serum dietary fatty acids and coronary heart disease risk – A nested case-control-study within the CARLA cohort

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Abstract *Background and Aims:* Diet is known to play a decisive role in the development of coronary heart disease (CHD). One factor believed to decrease lifetime risk of CHD is the consumption of omega-3 fatty acids. Yet, conclusive evidence regarding the potential cardioprotective effects of fatty acids is far from being reached. The present study aimed to provide further evidence on the association of serum fatty acid profiles with CHD risk.
Methods and Results: The CARdio-vascular Disease, Living and Ageing in Halle study (CARLA study) is an observational cohort study comprising an older adult's general population with a high level of cardiovascular risk factors. In a matched case–control design the serum fatty acid concentrations of 73 subjects with an incident fatal or nonfatal CHD event were compared to 146 controls matched for sex and age. Our data show that the participants of the CARLA study are underserved in unsaturated fatty acids with respect to current dietary recommendations. In addition, the ratio of omega-6 to omega-3 fatty acids was determined to be 8:1 which underlines the consumption of a Western-style diet enriched in omega-6 fatty acids. There were no significant differences in fatty acid patterns between cases and controls. Thus, no clear association of particular serum fatty acid levels with cardiovascular risk was found.
Conclusion: Our results support the conclusion that in populations with a homogenous low level of omega-3 polyunsaturated fatty acids consumption, serum fatty acid levels are not associated with CHD risk.

Introduction

Despite progress in prevention and therapy, coronary heart diseases (CHD) remain the leading cause of death worldwide [1]. It is widely believed that atherosclerosis is the main cause for CHD [2]. Plaques consisting of lipids harden and narrow the coronary arteries, reducing blood flow [3].

Dietary fat is a well-known modifiable risk factor for CHD [4,5]. Epidemiological evidence suggests that populations with a high intake of omega (n)-3 polyunsaturated fatty acids (PUFA), commonly found in certain fish, have a reduced risk of CHD [6–9]. The long-chain members of the n-3 family eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3) might achieve cardioprotective benefits by affecting atherogenesis [8]. Furthermore, the consumption of fats affects a large number of other known risk factors of CHD. It is well-

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documented that the consumption of n-3 PUFA has anti-arrhythmic actions as well as the reduction of blood pressure and plasma triglyceride levels especially in hypertensive persons or subjects with hypertriglyceridemia [10–15].

Accordingly several international bodies including the European Society for Cardiology and the American Heart Association recommend the regular consumption of n-3 PUFA from fish or fish oil capsules for CHD risk reduction [16,17]. This recommendation was followed by the evaluation of the effect of dietary preparations containing n-3 fatty acids on cardiovascular events and mortality by several clinical trials. However, recent intervention trials failed to show an effect of n-3 PUFA on cardiovascular outcomes which prompted a debate, whether or not n-3 PUFA are suitable for preventing CHD [9,10,18,19]. In this context the trials have been criticized for being underpowered, for ignoring baseline PUFA levels of study participants as well as bioavailability issues, and for overestimating compliance [9,10,18].

According to estimates less than 20% of the world population consumes the recommended daily n-3 PUFA doses [18,20]. In a global survey n-3 PUFA blood levels have been classified into one of four discrete groups based on the weight percentage values of EPA + DHA in plasma total lipids: very low ($\leq$2.9), low (>2.9–4.0), moderate (>4.0–5.2), and high (>5.2) [18]. At this, residents of Western countries (Europe and North America) have been identified to be at an increased risk of CHD due to low or very low blood levels of n-3 PUFA [18]. To our knowledge, no study has examined the associations of n-3 PUFA concentrations with cardiovascular events in extremely undersupplied populations characterized by very low n-3 PUFA blood levels.

Consequently, we performed a nested case–control study within the population based CARdiovascular Disease, Living and Ageing in Halle study (CARLA study) to analyse the association of 40 single fatty acids with risk of coronary heart disease. A lipidomic approach combined with multivariate data analysis, i.e. principal component analysis (PCA) was adopted for the investigation of potential diagnostic biomarkers in the serum of coronary heart disease patients.

Methods

Study design and setting

We used a matched case–control design with data from the CARdio-vascular Disease, Living and Ageing in Halle study (CARLA study), which is a prospective population-based cohort study of the elderly general population of the city of Halle in eastern Germany [21]. The CARLA cohort comprises 1779 participants, aged 45–83 years at baseline (967 men, 812 women). The baseline examination took place between December 2002 and January 2006. A multi-step recruitment strategy aimed to achieve a high response rate of 64% (after exclusion of individuals who were deceased prior to the invitation, had moved away, or were unable to participate due to illness). From March 2007 until March 2010 the first four-year follow-up examination was performed. The net sample (after exclusion of deceased or non-responding people) then comprised 1436 subjects (follow-up response 86%), consisting of 790 men and 646 women aged between 50 and 87 years, which were taken into account for analyses. In 2013 a second follow-up investigation was done.

In baseline and first follow-up the study participants underwent a detailed medical examination (ECG, echocardiography, anthropometric measures, blood pressure measurements, taking of blood samples) and a standardized, computer-assisted interview. The second follow-up investigation comprised a blood pressure measurement and a detailed computer-assisted interview. A more comprehensive account of the CARLA study can be found in Greiser et al. [21]. The study was approved by the Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg and by the State Data Privacy Commissioner of Saxony-Anhalt, and conforms to the principles outlined in the Declaration of Helsinki [22]. All participants gave written informed consent.

Selection of cases and controls

Cases were study participants with an incident first non-fatal acute myocardial infarction or with a fatal cardiovascular event (ICD-10 (international classification of disease, tenth revision) I21-I25). Incident first non-fatal acute myocardial infarction was defined based on self-reported physicians' diagnosis within the follow-up period. Cases with a fatal cardiovascular event were recorded within the mortality follow-up. The cause of death was defined as specified in the official death certificate compiled by the Federal Statistical Office: Initially, the cause of death was recorded by a medical doctor and subsequently reviewed by a certified coder at the Statistical State Office Saxony-Anhalt. Death was recorded from the beginning of the baseline investigation (December 2002) until the end of the second follow-up (October 2013). Each person with incident CHD (case, n = 73) was matched on sex, and age with two controls (n = 146). Controls were randomly selected from the eligible sample of study participants with no history of myocardial infarction, no percutaneous transluminal coronary angioplasty (PTCA), and no coronary artery bypass graft (CABG). Sample size calculation was based on the approach suggested by Lubin et al. [23]. We computed a required sample size of 73 cases when controls and cases are matched as 2:1 ratio. Here, we considered fatty acids from the n-3 fatty acid family as most relevant for the prediction of cardiac events and assumed a mean of 0.65 μmol/ml for the controls and 0.55 μmol/ml for cases (standard deviation for controls: 0.2, for cases: 0.225).

Exposure assessment

Serum fatty acid levels were determined by lipid extraction and subsequent gas chromatography analysis. The

serum lipids were trans-esterified with 500 μl methanolic HCl, 250 μl n-hexane and 500 μl internal standard (0.8 mg di-C17-phosphatidylcholine in 1 ml methanol with 0.2% butyl hydroxytoluene as antioxidant). After cooling-off, 500 μl n-hexane and 1 ml aqua dest. Were added. The upper hexane phase was evaporated with nitrogen. The fatty acid methylesters (FAME) were taken up in 60 μl n-hexane. An aliquot of 1 μl was injected on-column on a Varian CP 3800 gas chromatograph (Varian, Darmstadt, Germany) equipped with an Omegawax TM 320 column (0.32 mm internal diameter, 30 m length) (Supelco, Bellefonte, USA). The column temperature was 200 °C. In total 54 fatty acids in the range of C8 to C24 belonging to all known fatty acid families (saturated, n1, n3, n4, n5, n6, n7, n9, and n11) were quantified. However, we excluded 14 fatty acids for subsequent analyses due to a failure to achieve the detection limit in a high proportion (>90%) of study samples. The gas chromatographic analysis was performed in cooperation with the Institute of Biochemistry, Faculty of Veterinary Medicine, Leipzig University.

Potential confounders

Covariates used were measured during the baseline and first follow-up examinations. Potential confounders included, diabetes, the number of smoked cigarettes, cigars and pipes per day, alcohol intake, physical activity, triglycerides, fasting time, fasting time squared, and regular intake of lipid lowering drugs.

Hypertension was defined as the mean of measured systolic blood pressure ≥140 mmHg, and/or mean measured diastolic blood pressure ≥90 mmHg, and/or use of antihypertensive medication according to the anatomic therapeutic chemical classification system (ATC codes C02, C03, C07, C08, and C09). Diabetes was determined based on self-reported physician-diagnosed and/or use of antidiabetic medication (ATC code A10). Habitual physical activity over the previous 12 months was recorded using the Baecke questionnaire [24]. Being physically active was defined as giving an affirmative answer to the question “Do you play any sports?”. Subsequently, the hours per week a participant practiced sports derived from the questionnaire was considered as a covariate in the regression model.

Statistical analysis

Analyses were performed using R, version 3.2.3 (R Core Team, R Foundation for Statistical Computing, Vienna–Austria, www.R-project.org).

Missing values were imputed using the R package ‘mice’ with ten imputations. The percentage of missing data for further imputation in each variable was limited to 10% (low rate of missing values) [25]. In order to check for the assumption of missing values occurring at random we related the binary outcome of whether an observation was missing or not to the case–control status in the tow PUFA with the highest proportion of missing values (C22:1n9: 8.0% missing values and C18:4n3: 3.2% missing values) by means of a conditional logistic regression approach. We found in both cases there was no evidence for an association of missing values with the case–control status ($p = 0.64$ and 0.26, respectively).

Fatty acids (log-transformed and standardized) were related to the case–control status by means of crude and adjusted conditional logistic regression. We took the ‘logit’ of the values

$$\ln\frac{p}{1-p'}$$

where p refers to the percentage of the respective fatty acid. Here, the odds ratios refer to the logarithmic change in the percentage frequency of the respective fatty acid in relation to the remaining fatty acids, i.e. a 2.7-fold (Euler’s number) increase in the quotient of the relative frequency of fatty acids in relation to the remaining fatty acids corresponds to the odds ratio as they are reported here. Analyses were replicated for quartile-based categories of each fatty acid (lowest quartile as reference) with conditional logistic regression.

We used principal component analysis (PCA) to derive key components of measured fatty acids. These key components standardize the measurement of fatty acids to several composite parameters that explain most of the variance of the set of considered fatty acids. This method avoids multiple significant and redundant results when highly correlated parameters are associated with the same outcome. The extracted components were subsequently used as predictor variables in logistic regression analyses (principal component regression). We assessed the adequacy of the models by plotting the deviance residuals. Residuals showed a random pattern as expected when model assumptions hold.

To correct for multiple testing we identified the number of independent (orthogonal) components of a further principal component analysis that explained 95% of the variance ($n = 23$). This number was subsequently used to correct the level of significance by means of the Šidak correction.

Results

Study population

We identified 73 subjects as incident cases and matched them with 146 controls. Differences between cases and controls were seen in cardiovascular risk factors: diabetes prevalence, frequency of current smoking, alcohol consumption, physical activity, level of triglycerides, and intake of statins (Table 1). Consequently, multifactorial analyses were adjusted for these factors.

Fatty acid levels and coronary heart disease risk

There were no or only small differences in the concentrations of fatty acids between cases and controls, with a trend for higher levels of nearly all fatty acids in the case group (Table 2). Noteworthy differences in this raw

Table 1 Characteristics of incident CHD cases and controls (variables assessed up to 4 years before diagnosis) (statistical analysis performed to obtain P values: *t*-test for continuous and Chi-square test for categorical variables).

	Cases (N = 73) N [%] or Mean [SD]	Controls (N = 146) N [%] or Mean [SD]	P-value
Age [years]	69.1 [10.9]	69.1 [10.9]	1.000
Sex, female	21 [28.8]	42 [28.8]	1.000
Education [years]	14.7 [2.9]	14.4 [2.6]	0.431
Hypertension	59 [80.8]	115 [78.8]	0.722
Diabetes mellitus	22 [30.1]	23 [15.7]	0.013
HbA1c	6.1 [1.0]	5.7 [0.6]	0.003
Current smoker	18 [24.7]	29 [19.9]	0.512
Alcohol consumption [g/day]	10.5 [13.8]	15.4 [21.1]	0.040
Sport activity	55 [75.3]	96 [65.8]	0.148
BMI	28.2 [4.8]	27.6 [4.3]	0.413
Cholesterol [mmol/l]	5.6 [1.2]	5.4 [1.0]	0.248
HDL [mmol/l]	1.3 [0.4]	1.4 [0.4]	0.024
LDL [mmol/l]	3.5 [1.0]	3.4 [0.9]	0.418
Triglyceride [mmol/l]	2.5 [2.3]	1.8 [1.0]	0.011
Use of lipid lowering drugs	13 [17.8]	21 [14.4]	0.510
Fasting time [hours]	4.2 [1.7]	3.7 [1.5]	0.024

analysis were observed in the family of omega-9 fatty acids (n9) and more specifically in oleic acid (C18:1n9) which was the most prominent omega-9 fatty acids in our population. To further describe the relationship between fatty acid levels and CHD risk, we calculated the adjusted ORs (95% CIs) of CHD risk for fatty acid profiles (Fig. 1). None of the individual or total fatty acid levels were associated with increased CHD risk in the model after adjustment for confounders.

Additional we checked the association of quartile-based categories of each fatty acid (lowest quartile as reference) with case–control status. However, there was only a trend indicating higher risk for being a case among patients with higher fatty acid levels, compared with those in the lower fatty acid quartiles. Despite this, there was no significant difference in CHD incidence across the fatty acid quartiles.

Principal component analysis revealed that three components were needed to best explain the variance between considered serum levels of fatty acids (supplemental figure S1). Explained variance of fatty acids was low and ranged from 14.5% for the first factor (RC1) to 7.9% for the third factor (RC3). However, as seen in supplemental figure S1, no clear pattern emerged in RC1, RC2, and RC3. The cumulative proportion of explained variance with three components was only 37%.

Moreover, after multiple adjustment the significance of the components in association with case/control status was weakened by a considerable amount of statistical uncertainty as it is indicated by wide corrected confidence intervals (OR RC1: 1.43, 95% cCI: 0.61–3.37; OR RC2: 0.90, 95% cCI: 0.53–1.52; OR RC3: 1.09, 95% cCI: 0.59–2.04).

Discussion

Coronary heart disease is caused by a vascular occlusion which results from fatty deposits on the inner vessel walls. Since lifestyle factors, such as the diet, are known to contribute to the development of CHD, the preventive potential of nutritional fats and fatty acids has extensively discussed. Numerous studies have hitherto investigated the role of different types of fat in CHD, however, it is still not clear whether a diet enriched in particular fatty acids is suitable in the prevention of primary CHD events.

The present study aimed at providing population-based data on serum fatty acid profiles and their association with myocardial infarction, taking as a basis serum samples and medical documentation of the CARLA cohort. The CARLA study is an observational cohort study which comprises an elderly general population with a high level of cardiovascular risk factors (e.g. hypertension, diabetes, increased cholesterol and triglyceride levels) exceeding the risk level of other western European populations [21]. In a matched case–control design the serum fatty acid profiles at baseline of persons with an incident CHD event during follow-up were compared to controls being matched for sex and age.

As expected, baseline risk factors for CHD were more prevalent in cases compared to controls. Cases were more likely to have diabetes, take lipid-lowering medication, be smokers, and have a lower average alcohol intake.

The average weight percentage values of EPA + DHA (cases: 2.77; controls: 2.75) were found to be very low. Our data, therefore, show that the participants of our subsample of the CARLA study are underserved in unsaturated fatty acids with respect to current dietary recommendations. In addition, the ratio of omega-6 (n6) to omega-3 (n3) PUFA in serum samples was detected to be 8: 1 which underlines the consumption of a Western-style diet enriched in omega-6 fatty acids. Overall, there were no significant differences in fatty acid patterns between cases and controls. For that reason no clear association of particular serum fatty acid levels with CHD risk was found.

Data from previous studies indicate a beneficial effect of fish consumption or rather the intake of omega-3 PUFA supplements on CHD risk [11,26–28]. Moreover, several trials unanimously report an inverse association of omega-3 PUFA blood levels with CHD mortality [10,19,29]. With

Table 2 Serum fatty acid profiles [μmol/ml] in cases and controls (statistical analysis performed to obtain P values: *t*-test).

	Cases (N = 73) Mean (95% CI)	Controls (N = 146) Mean (95% CI)	P value
Saturated fatty acids	5.461 (4.841; 6.081)	4.716 (4.506; 4.926)	0.026
C14:0	0.249 (0.202; 0.297)	0.196 (0.177; 0.215)	0.041
C15:0	0.038 (0.032; 0.044)	0.030 (0.028; 0.033)	0.015
C16:0	3.979 (3.513; 4.445)	3.405 (3.249; 3.560)	0.022
C18:0	1.076 (0.972; 1.179)	0.976 (0.938; 1.015)	0.076
C20:0	0.022 (0.020; 0.024)	0.020 (0.019; 0.021)	0.053
C22:0	0.039 (0.037; 0.042)	0.038 (0.036; 0.039)	0.258
C23:0	0.016 (0.015; 0.018)	0.016 (0.015; 0.017)	0.463
C24:0	0.029 (0.027; 0.031)	0.029 (0.028; 0.030)	0.745
Unsaturated fatty acids n-3	0.633 (0.584; 0.683)	0.589 (0.559; 0.619)	0.109
C18:3n3	0.128 (0.105; 0.150)	0.108 (0.098; 0.117)	0.097
C18:4n3	0.006 (0.005; 0.008)	0.005 (0.005; 0.006)	0.155
C20:3n3	0.004 (0.003; 0.004)	0.003 (0.003; 0.004)	0.190
C20:4n3	0.016 (0.013; 0.019)	0.014 (0.013; 0.015)	0.163
C20:5n3	0.139 (0.125; 0.153)	0.135 (0.124; 0.145)	0.657
C22:5n3	0.073 (0.067; 0.079)	0.071 (0.067; 0.074)	0.484
C22:6n3	0.267 (0.249; 0.285)	0.253 (0.240; 0.266)	0.214
Unsaturated fatty acids n-6	4.996 (4.752; 5.240)	4.819 (4.665; 4.974)	0.209
C18:2n6	3.866 (3.658; 4.075)	3.697 (3.565; 3.829)	0.159
C18:3n6	0.059 (0.052; 0.065)	0.058 (0.054; 0.062)	0.766
C20:2n6	0.036 (0.033; 0.038)	0.033 (0.032; 0.035)	0.073
C20:3n6	0.194 (0.179; 0.210)	0.184 (0.176; 0.193)	0.262
C20:4n6	0.815 (0.772; 0.858)	0.825 (0.792; 0.858)	0.714
C22:4n6	0.026 (0.018; 0.035)	0.022 (0.021; 0.024)	0.361
C22:5n6	0.017 (0.015; 0.018)	0.016 (0.015; 0.017)	0.465
Unsaturated fatty acids n-7	0.725 (0.626; 0.823)	0.647 (0.604; 0.689)	0.150
C16:1n7	0.416 (0.351; 0.481)	0.374 (0.344; 0.405)	0.251
C18:1n7	0.283 (0.248; 0.318)	0.248 (0.234; 0.262)	0.068
C18:2n7	0.009 (0.008; 0.010)	0.008 (0.007; 0.008)	0.098
C20:3n7	0.016 (0.014; 0.017)	0.016 (0.016; 0.017)	0.437
Unsaturated fatty acids n-9	3.999 (3.499; 4.499)	3.407 (3.222; 3.593)	0.029
C16:1n9	0.053 (0.047; 0.059)	0.046 (0.043; 0.049)	0.341
C18:1n9	3.798 (3.312; 4.284)	3.222 (3.042; 3.402)	0.029
C18:2n9	0.012 (0.011; 0.014)	0.011 (0.010; 0.011)	0.065
C20:1n9	0.033 (0.029; 0.037)	0.028 (0.026; 0.030)	0.032
C20:2n9	0.003 (0.003; 0.004)	0.003 (0.003; 0.003)	0.297
C20:3n9	0.019 (0.017; 0.022)	0.019 (0.018; 0.021)	0.988
C22:1n9	0.007 (0.006; 0.008)	0.005 (0.004; 0.005)	0.002
C24:1n9	0.074 (0.070; 0.078)	0.075 (0.072; 0.078)	0.783
Other unsaturated fatty acids			
C14:1n5	0.017 (0.012; 0.022)	0.014 (0.011; 0.016)	0.231
C18:1n5	0.019 (0.015; 0.022)	0.015 (0.014; 0.017)	0.092
C18:2n4	0.011 (0.009; 0.013)	0.009 (0.008; 0.010)	0.068
C16:4n1	0.041 (0.036; 0.047)	0.034 (0.032; 0.036)	0.014
CLAc9t11	0.003 (0.002; 0.003)	0.003 (0.002; 0.003)	0.561
C20:1n11	0.011 (0.008; 0.013)	0.008 (0.007; 0.008)	0.045
Total fatty acids	15.938 (14.499; 17.376)	14.283 (13.734; 14.833)	0.035

respect to myocardial infarction the picture is much less clear. The available data do not allow at present a conclusive appraisal of the role of PUFA of both the omega-3 and the omega-6 family in a primary prevention setting. The results of studies investigating the influence of fish consumption on myocardial infarction vary from a beneficial impact to a tendency for an inverse association or even neutral effects [30–32]. Likewise no clear association between PUFA blood levels and myocardial infarction risk has been found, so far [33,34]. Nevertheless, in accordance to our data, a previous study investigating a Dutch population consuming a diet high in saturated fatty acids and low in PUFA, which corresponds to the CARLA cohort, observed no relation between dietary fatty acid classes and CHD risk [31]. It might therefore be speculated that the cardioprotective effects of omega-3 PUFA can be only observed at a high level of intake. In fact, omega-3 PUFA have been described to reduce blood pressure as well as serum triglyceride levels when administered in doses of 3 g/day or more [11,35–37]. Furthermore, in a current meta-analysis a CHD risk reduction benefit of omega-3 PUFA was solely seen for high risk individuals with respect to levels of triglycerides (above 1.71 mmol/l) and LDL cholesterol (above 3.38 mmol/l) [12].

Our study has several strengths. We used fatty acid serum level instead of information about dietary intakes of fatty acid. The measurement of circulating fatty acids excludes the possibility of self-reporting bias at this

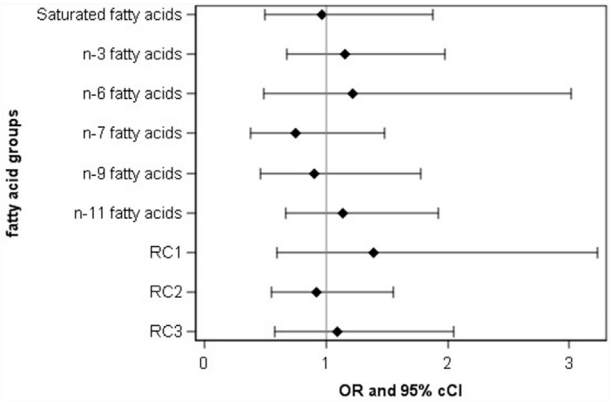

Figure 1 Conditional fatty acid specific logistic regression analysis: Odds ratios with 95% confidence intervals corrected for multiple testing. Models were adjusted for diabetes, the number of smoked cigarettes, cigars and pipes per day, physical activity, alcohol intake, triglycerides, fasting time, fasting time squared, and regular intake of lipid lowering drugs.

objectively reflecting the habitual PUFA consumption that influences tissue levels [10,38]. Moreover, this method allows for the separate evaluation of each individual fatty acid. Further strengths of our study include the prospective sampling of cases, the large number of fatty acids analyzed comprising all fatty acid families, and the performance of a pattern identification by means of PCA.

Potential limitations to be considered are the small sample size, especially due to the low number of incident cases as well as the homogeneity of samples with a small range of fatty acid concentration values, probably because of less variation in dietary habit. Thus, our results may not be directly applied to populations which fulfill current dietary guidelines with respect to PUFA consumption. Fatty acid levels were measured once at baseline and potential dietary fluctuations during follow-up could not be taken into account. Study participants were fasted before blood collection. Nevertheless, it cannot be completely excluded that measured data might be influenced by eating behavior immediately prior sampling. Our biosamples were stored for up to 14 years at −80 °C before analysis. However, serum fatty acids have been shown to be very stable at sub-zero temperatures. In fact, no significant PUFA degeneration was found in serum samples stored for up to 10 years at −80 °C [39]. For that reason we can be convinced that the data collected in our fatty acid composition study properly reflects reality.

In summary, our data suggest that in populations with a homogenous low level of omega-3 PUFA consumption serum fatty acid levels are not associated with the CHD risk.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2018.10.006.

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