Influence of blue mussel (*Mytilus edulis*) intake on fatty acid composition in erythrocytes and plasma phospholipids and serum metabolites in women with Rheumatoid Arthritis

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Keywords: rheumatoid arthritis; seafood; *Mytilus edulis*; n-3 LC PUFA; erythrocyte fatty acids, metabolomics

Abbreviations:

RA-rheumatoid arthritis, MIRA-Mussels, inflammation and Rheumatoid Arthritis trial, PCA-Principal component analysis, OPLS-DA-Orthogonal Projections to Latent Structures with Discriminant Analysis, OPLS-EP-OPLS with effect projections, ^{1}H-NMR- ^{1}H-Nuclear Magnetic Resonance, DAS28-disease activity score 28, CVD- cardiovascular disease, BMI- body mass index, TAG- triacylglycerols, HDL- high density lipoprotein, LDL- low density lipoprotein (LDL), CRP- C-reactive protein, n-3 -omega-3, LC PUFAs- long chain polyunsaturated fatty acids, EPA- eicosapentaenoic acid, DHA- docosahexaenoic acid, ARA- arachidonic acid, DGLA -dihomo-gamma-linolenic acid.

Summary

An intake of 375g blue mussels for 11 weeks decreased disease activity in women (*n*=23) with rheumatoid arthritis in a randomized cross-over trial. This study investigates potential causes of the decreased disease activity by analysing fatty acid composition in erythrocytes and plasma phospholipids and ^{1}H-NMR serum metabolites. Blue mussel intake resulted in significant increases in EPA and DHA at the group level but not for all individuals. However, multivariate modelling of the fatty acid profile in erythrocytes resulted in all samples being correctly classified to either the blue mussel diet or control diet. To conclude, modelling fatty acids in erythrocytes may be a better biomarker for seafood intake than only EPA and DHA content. The change in fatty acid pattern in erythrocytes could be related to reduction in disease activity, although it cannot be excluded that other factors than omega-3 fatty acids potentiate the effect.

1. Introduction

Negative associations between intake of omega-3 (n-3) long chain polyunsaturated fatty acids (LC PUFAs) and risk or risk factors of cardiovascular disease (CVD) and inflammation are generally seen [1-3]. Seafood, especially fatty fish, is the main dietary source of the bioactive n-3 LC PUFAs eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA). Fish as a source of n-3 LC PUFAs is not economically nor environmentally sustainable [4]. In contrast, blue mussel farming is beneficial for the environment since excessive nitrogen is removed at harvest and eutrophication of the sea is reduced [5]. Blue mussels contain approximately 0.7 g EPA and DHA/100 g boiled mussel meat and are rich in nutrients such as zinc, selenium, riboflavin and carotenoids [6]. This makes blue mussels a potentially significant source of both EPA and DHA and other nutrients that could influence the metabolome and improve overall health.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by systemic inflammation and joint damage. RA affects 0.5–1% of the population globally, predominantly women [7]. We have previously shown, in the randomized cross-over trial Mussels, Inflammation and RA (MIRA), that disease activity, fatigue, pain and general health in RA is improved by a dietary intervention with blue mussels [8]. Briefly, intake of blue mussels led to a significant reduction in disease activity score 28 (DAS28)-C-reactive protein (CRP), with a trend towards significance for reduced CRP and reduced white blood cell count. It is not clear what the mechanisms of action of the dietary intervention were, or if the intake of n-3 LC PUFAs from the blue mussels plays a role. N-3 LC PUFAs have been

shown to be useful in treating some symptoms of RA [9, 10], presumably through their anti-inflammatory actions [11].

Thus, the aim of this study was to confirm compliance in the MIRA study, with the gold standard method the omega-3 index (EPA plus DHA in red blood cells). Further, we wanted to explore if intake of blue mussels in patients with RA leads to changes in fatty acid composition in erythrocytes and plasma phospholipids and/or in metabolites (detected by NMR-metabolomics), compared to a control diet, in order to understand the beneficial health effects seen in the MIRA study. To our knowledge this is the first human intervention study that has investigated the effects of blue mussel intake on fatty acid status in both erythrocytes and plasma phospholipids and NMR-metabolomics.

2. Patients and Methods

2.1 Subjects and experimental design

Participating patients and experimental design were described in detail in our previous publication [8]. Briefly, 39 women (25-65 years of age) with established RA and DAS28 >3.0 were allocated to start with either the blue mussel or control diet in a randomized, single-blinded cross-over intervention. Twenty-three women completed both dietary periods (eleven weeks, respectively, with an eight-week washout period in between) and blood samples from those women were analysed for fatty acids and metabolites in the current paper. Compliance was evaluated by a self-reporting number of dishes consumed and 24-h dietary recalls conducted by telephone midway through each dietary period. Subjects' baseline characteristics have been presented in detail elsewhere [8].

All procedures were conducted according to the Declaration of Helsinki and approved by Gothenburg Regional Ethical Review Board (25 May 2015/Dnr 230-15), and all participants signed an informed consent. The trial was registered at https://register.clinicaltrials.gov as NCT02522052.

2.2 Analysis of fatty acid

2.2.1 Sample collection and handling

Fasting blood samples were collected into 6 mL lithium heparin vacutainer tubes (BD Vacutainer®, Heparin tubes, Franklin Lakes, NJ, USA) and centrifuged at room temperature

(913 x g for 10 min) within three hours of collection and the plasma isolated. Erythrocytes were then washed with PBS-sterile liquid (VWR life science) and centrifuged twice (350 x g for 10 minutes, room temperature, low brake). All plasma and erythrocyte samples were stored in a -80º C freezer until analysis.

2.2.2 Fatty acid analysis

Fatty acids in erythrocytes and in plasma phospholipids were analysed by gas chromatography as described previously [12]. In short, total lipid was extracted into chloroform:methanol. Phosphatidylcholine, the major phospholipid in plasma, was isolated by solid phase extraction. Fatty acid methyl esters were formed from the erythrocyte and plasma phospholipid extracts by heating with methanolic sulphuric acid. Fatty acid methyl esters were separated by gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column using the settings and run conditions described elsewhere [12]. Fatty acid methyl esters were identified by comparison with run times of authentic standards. Data are expressed as weight % of total fatty acids. The omega-3 index was calculated by adding percentage of EPA and DHA in erythrocytes and is presented as mean (s.d.).

2.3 NMR-analysis of serum metabolites

2.3.1 Sample collection and handling

Fasting blood samples were collected in 5 mL glass vacutainer tubes (BD Hemogard TM BD Vacutainer®), turned approximately 5 times, allowed to clot for 5-10 min at room temperature and then at 8°C for 30 min. Tubes were centrifuged in room temperature at

2600 x g for 10 min. The aliquoted serum was kept at 8°C for a maximum of 3 h and then stored at -80°C until analysis. Prior to ^{1}H-NMR analysis, serum samples were thawed for 60 min at 4°C. Then 100 µL serum was mixed with 100 µL phosphate buffer (75 mM Na_2HPO_4, 20% D_2O, 0.2 mM imidazole, 4% NaN_3, 0.08% TSP-d_4, pH 7.4) in a deep well plate. 180 µL sample mix was transferred to 3.0 mm NMR tubes (Bruker BioSpin, 96 sample racks for SampleJet) using a SamplePro liquid handling robot (Bruker BioSpin, Rheinstetten, Germany). Samples were kept at 6°C in the SampleJet sample changer until analysis.

2.3.2 NMR spectroscopy

^{1}H-NMR spectra were acquired on a Bruker Avance III HD spectrometer (800 nm) with a 3 mm TCI cryoprobe and a cooled (6°C) SampleJet for sample handling. All ^{1}H-NMR experiments were performed at 298 K. NMR data (1D perfect echo with excitation sculpting for water suppression) were recorded using the Bruker pulse sequence 'zgespe'. The spectral width was 20 ppm, the relaxation delay 1.3 s, the acquisition time 2.04 s and a total of 128 scans were collected into 64 k data points resulting in a measurement time for each sample of 12 min 4 s. All data sets were zero filled to 128 k and an exponential line-broadening of 0.3 Hz was applied before Fourier transformation. All data processing was performed with TopSpin 3.2pl6 (Bruker BioSpin, Rheinstetten, Germany) and TSP-d_4 was used for referencing. Chenomx NMR suite 8.31 (Chenomx Inc., Edmonton, Canada) was used for annotation with the aid of the Human Metabolome Database (HMDB) [13].

2.3.3 Data-processing

^{1}H-NMR spectra were aligned using icoshift [14] and manual integration of peaks was performed to a linear baseline on all spectra in parallel. In total 316 peaks were integrated within the chemical shift range of 0.721 – 8.358 ppm; none of the variables were excluded. Data were non-normalized.

2.4 Statistical Methods

2.4.1 Multivariate methods

All multivariate analyses were performed using SIMCA software v.15.0 (Umetrics AB, Umeå, Sweden) and no samples were excluded in any of the analysis. Principal component analysis (PCA) models and Orthogonal Projections to Latent Structures (OPLS) were used to explore clustering patterns of observations, trends in the data in relation to known factors and outliers. OPLS models include not only x-values such as fatty acid data but also y-values i.e. additional known factors that could influence the data such as body mass index (BMI), triacylglycerols (TAG), high density lipoprotein (HDL), low density lipoprotein (LDL), age and sex. The presented OPLS-models only include y-values that have a CV-ANOVA $p < 0.05$ for the model. Separation of classes and variables related to separation in the data according to classification of diet (*blue mussels vs control diet*) were evaluated using an Orthogonal Projections to Latent Structures with effect projections (OPLS-EP), where delta values between periods (post mussels-post control) were used since the samples were paired. In addition, OPLS with Discriminant Analysis (OPLS-DA), where delta values for each period (post-pre) were used (n=46), was performed. Cross-validation groups were set to 23 (equal to the number of study participants) and were based on individual ID, so that all four samples from one individual were left out in one cross-validation round. The validity of

OPLS-DA models was assessed using permutation tests (n=999). Validated prediction models for performance are presented using Receiver Operating Characteristics (ROC) Curve for OPLS-DA models. Also, to further test the model quality, test sets (5-6 individuals) was selected by computerized randomization. The remaining 17-18 individual's samples for each set were used as a training set and the test set was projected onto the training set model. This was repeated ten times and median values for ROC curve and correct classification were calculated. Also, cross-validated predictive residuals (CV-ANOVA) visual comparison between scores and cross-validated scores, the cumulative amount of explained variation in the data summarized by the model (R2X[cum] and R2Y[cum]), and the predictive ability of the model (Q2[cum]) are presented.

Class discriminating variables of interest from the OPLS-EP and OPLS-DA models were selected if loadings w ≥± 0.1 and if they were among the thirty highest VIP-scores and further assessed by univariate analysis.

2.4.2 Univariate methods

Statistical analyses were performed using SPSS version 25 (SPSS Inc., Chicago, IL, USA). Treatment effect of the dietary intervention for all fatty acids was evaluated using a mixed effect analysis of covariance (ANCOVA) model with treatment (intervention or control), period and sequence as fixed effects subject nested in sequence as a random effect. Baseline values for the fatty acid were included as a covariate. Least squares means were calculated for each treatment as well as the difference (Break vs No break) and are presented with 95% confidence intervals and p-values. Wilcoxon signed rank test was used to evaluate metabolites driving the separation in OPLS-DA and OPLS-EP models. Spearman's

196 correlation analysis was performed to evaluate associations between outcome variables
197 DAS28, CRP, TAG and HDL and fatty acids between all samples (n=92), adjusted for age and
198 BMI. In this explorative study data are presented as mean (s.d.) with significance set at
199 α=0.05, i.e. it is not corrected for multitesting.

200

201

3. Results

3.1 Fatty acid composition in relation to health biomarkers

The PCA plots in **Figure 1A** and **B** show erythrocyte fatty acids (%) (model 1, **Table 1**) and plasma phospholipid fatty acids (%) (model 2, Table 1). Each individual is represented by four samples (n=92), i.e. before and after each dietary period. The patterns of fatty acids differed slightly between the erythrocytes and phospholipids (Figure 1 A and B). In erythrocytes palmitic (16:0), myristic (14:0) and n-3 fatty acids clustered and in plasma phospholipids stearic (18:0) and n-3 fatty acids clustered. In OPLS models (model 4 and 5, Table 1) TAG, HDL and ApoA1 were included as y-values and all had a CV-ANOVA correlation $p<0.001$ in the model. BMI and TAG correlated strongly ($r=0.345$, $p= 0.000112$, not shown in figure).

3.2 Influence of blue mussel intake on fatty acid composition

OPLS-EP analysis showed that the fatty acid pattern after blue mussel intake and control diet differed for erythrocyte fatty acids (model 7, table 1 and **Figure 2A**), but not for plasma phospholipids, where only a weak OPLS-EP model could be created when forcing a two component model (model 8, table 1). This was also confirmed by the OPLS-DA models created for delta values for the dietary periods i.e. post-pre-values for fatty acids (model 10 and 12, table 1). The plasma phospholipid model had a low quality indicating that plasma phospholipid pattern is a poor marker for blue mussel intake (model 11, table 1, **Supplemental figure 1B, 2B and supplemental table 1**). On the contrary, the erythrocyte model had a high quality (model 10, table 1), which was confirmed both by permutation

tests and ROC curve (**Supplemental figure 1A and 2A**) and by the use of test and training sets (supplemental table 2). In addition, all individuals were correctly classified according to intervention diet **(Figure 2B)** in the OPLS-DA erythrocyte model.

3.3 Influence of blue mussel intake on individual fatty acids in relation to health outcomes

Fatty acids in erythrocytes and plasma phospholipid are presented in **Table 2**. EPA and DHA were both increased in erythrocytes after the blue mussel diet, compared to the control diet, resulting in a decrease in n-6 PUFA arachidonic acid (ARA; 20:4 n-6) and dihomo-gamma-linolenic acid (DGLA; 20:3 n−6) as well as a small decrease in saturated fatty acids and the mono unsaturated fatty acid palmitoleic acid (16:1 n-7). The omega-3 index was 7.7 (1.0) % and 8.4 (1.0) % before and after blue mussel diet, respectively and 7.8 (1.0) % and 7.6 (1.0) % before and after control diet, respectively. The omega-3 index did not differ between the two baseline measurements neither for the whole group nor for the two groups that started with different diets. In plasma phospholipids, n-3 fatty acids increased after the mussel diet, mainly due to an increase in EPA, while saturated fatty acids decreased. Higher EPA and DHA (%) after the blue mussel diet compared to the control diet was seen in 21 of 23 individuals in erythrocytes, and in 18 of 23 individuals in plasma phospholipids. Comparing within periods, the expected increase in EPA and DHA (%) after the blue mussel diet was confirmed in 20 of 23 individuals in erythrocytes (**Figure 3**) and 17 of 23 individuals in plasma phospholipids. Spearman's correlation test showed negative associations between white blood cell count and EPA (r= -0.45, p=0.045), ESR and EPA (r=-0.53, p=0.015) and docosapentaenoic acid (DPA) (r=-0.50, p=0.025), after adjusting for BMI and age.

3.4 Metabolite patterns in relation to health biomarkers

Glucose and lipids were the main factors behind the first component (17.2% of the explained variation (R^2X)) in the PCA model (model 3, table 1). The individual with the highest BMI (BMI>35 kg/m^2) was an obvious outlier in this component (**Figure 4A**). In the third component (13.8% of the explained variation (R^2X)) (**Figure 4B**), one individual with high white blood cells (>10) and high platelet count (>500) was an outlier. The third component was mainly driven by proline, phenylalanine, and leucine with arginine, glutamine, and glucose on the opposite side. In the OPLS model for metabolites (model 6, table 1) BMI, TAG and platelet count were included as y-values, all with a CV-ANOVA $p<0.01$.

3.5 Metabolomics approach to separate between diets

None of the components in the PCA model (model 3, table 1), were related to the dietary intervention. However, it was possible to model the data using OPLS-EP (model 9, table 1) and a forced two component OPLS-DA model (model 12, table 1) to separate between the diets. However, none of these were good models for separating the blue mussel diet from the control diet. This was the conclusion from the permutation test (**Supplemental figure 1C**), test and training sets (**Supplemental table 3**) and the predictions for all individuals in the OPLS-EP model (**supplemental figure 3)**. Discriminating metabolites are presented in **Supplemental table 4**. The OPLS-EP model was driven by a difference in glucose and a peak including proline and one unidentified metabolite, but a significant difference was only found between the values post diets and not between pre and post blue mussel intake with univariate statistics (Supplemental table 4). Excluding glucose from the model only improved it slightly (data not shown). Discriminating metabolites from the OPLS-DA model

(model 12, table 1) confirmed the peak for proline and one unidentified peak, but not glucose.

4. Discussion and Conclusions

4.1 Descriptive results of fatty acids in women with RA

The fatty acid patterns in erythrocytes and plasma differed, as expected, but both confirmed the well-known relation between high EPA and DHA and high HDL and low TAG. The percentage of erythrocyte palmitic acid (16:0) was slightly higher in our study than what studies in healthy subjects have reported [15-17]. However, the fatty acid pattern was similar to data from women and patients with RA reported in other studies [18, 19]. Differences in erythrocyte composition between RA patients and healthy controls have been reported: a Korean study found that erythrocyte levels of α-linolenic acid (18:3 n-3), EPA, the omega-3 index (erythrocyte levels of EPA plus DHA), 18:2 n-6, and 18:1 n-9 were lower, while 18:3 n-6, 14:0, 16:0, 18:0, and 16:1 n-7 were higher in patients with RA [18]. In addition, palmitic and stearic acids in plasma phospholipids have been positively associated with adiposity, TAG, inflammation biomarkers and insulin resistance in nondiabetic older adults [20]. To our knowledge there are no studies that have investigated if the metabolic syndrome, obesity or RA influence the composition of fatty acids in erythrocytes, controlling for diet. In addition, the participants in our study had rather high EPA and DHA in erythrocytes at baseline compared to healthy men and women [21] and obese and normal weight adolescents [22], but values were similar to overweight and obese women with RA from Korea [22]. This

indicates that our participants ate fish and shellfish several times a week habitually, which was also confirmed in a food frequency report at baseline [8]. We have previously reported that fish and shellfish intake was higher among women with RA in the Gothenburg area compared to Sweden in general [23].

4.2 Intervention results of fatty acids in women with RA

The percentage of n-3 fatty acids in erythrocytes and EPA in plasma phospholipids increased at group level during the blue mussel diet compared to the control diet, despite rather low intake of n-3 fatty acids from the mussels (about 0.3 g/day). The n-3 fatty acids in erythrocytes increased about 0.66 % of total fatty acids, in a seemingly dose dependent increment in EPA and DHA content - similar to results from other studies with intake of fish and fish oil capsules [16, 21].

A decrease in the n-6 PUFAs ARA (20:4 n-6) and DGLA (20:3 n−6), a derivative of gamma linolenic acid, which in turn is a derivative of linoleic acid, as well as a small decrease in saturated fatty acids and palmitoleic acid (16:1 n-7) was also seen after blue mussel intake. The increment in EPA and DHA and the decrease in DGLA, ARA and palmitoleic acid is similar to what others have shown in RA patients after intake of long chain n-3 fatty acids in capsules [24, 25]. Since ARA, DGLA and EPA all are substrates for cyclooxygenase and lipoxygenase, giving rise to different series of prostaglandins and leukotrienes, more or less inflammatory and the changes in erythrocyte membranes could accordingly have an effect on inflammation. In addition, higher DGLA is associated with higher CRP [26]. Thus, the

decreased ARA and DGLA in combination with increased EPA and DHA might explain the trend towards a decreased CRP in the MIRA study. Negative correlations between EPA and health outcomes, such as white blood cell count and ESR, indicate that the n-3 LC-PUFA might play a role in the disease activity reducing effects seen in the MIRA study.

As described previously, the percentage of n-3 fatty acids in the erythrocytes as well as EPA in plasma phospholipids increased for the group during the blue mussel diet compared to the control diet. It has beenreported that the anti-inflammatory effect (reduced CRP) from fish oil in patients with RA increased when combined with a lacto-vegetarian diet low in ARA [16]. Further, daily consumption of DHA decreased erythrocyte ARA and led to a decline in the sum of tender and swollen joints [25]. Thus, not only the increase of EPA and DHA in serum is important to monitor but also the overall fatty acid composition changes from the total dietary intake. The importance of analyzing the fatty acid pattern was also confirmed in our data, since the expected changes in EPA and DHA were not seen in all individuals, indicating either poor compliance or that the biomarker was influenced by other factors. Genetic differences, metabolic disturbance, body weight, sex, physical activity, EPA and DHA at baseline and other dietary factors may influence erythrocyte composition and the omega-3 index may have limitations as a dietary biomarker in patients with RA, obesity or other diseases [21, 27]. Our results show that modelling fatty acids in erythrocytes could increase the precision, compared to only EPA and DHA content, when used as a biomarker for seafood (model 7 and 10, Table 1).

4.3 Descriptive results of metabolomics in women with RA

Chronic inflammation may cause metabolic changes, especially in the context of cachexia-associated catabolism [28], but none of the disease related markers such as CRP or DAS28 were significantly associated in the OPLS model in our study. Few studies have reported metabolites discriminating between patients with RA versus controls [29-31] and none report important descriptive data such as sex, BMI or diet, which are well known to influence the metabolome [32-34]. Lactate, tyrosine, phenylalanine and glycerol have been reported to have a strong relation to BMI [32], but also 3-hydroxybutyrate, glucose, isoleucine, leucine, pyruvate and glutamine concentrations are related to BMI [32], confirming that BMI should be taken into consideration in all metabolomics studies performed in the context of dietary exposure or dietary change. Thus, it cannot be ruled out that the metabolites described as discriminating for RA in these studies reflect differences in BMI, body composition and diet, more than the disease itself. However, our PCA analysis confirmed that BMI had a large impact on the metabolites but also white blood cell count and platelet count influenced the model, indicating that metabolomics could potentially be useful in describing disease activity.

4.4 Intervention results of metabolomics in women with RA

Since there was no good model separating the diets based on the features observed by NMR metabolomics, the results related to discriminating metabolites must be interpreted with caution

In short, the main finding was that glucose was lower after the blue mussel diet compared to the control diet but did not decrease during the blue mussel period. Thus, we cannot ascribe the effect to the blue mussel intake, but this could be of interest to investigate

360 further. In addition, proline (in a peak including also an unidentified metabolite) was lower
361 after the blue mussel diet. It has been shown previously that dietary proline and proline
362 concentrations in serum correlate [36]. However, the intervention foods blue mussels and
363 chicken do not differ significantly in proline content [37]. Thus, it could be speculated that
364 proline concentration reflects the known effect of inflammation on glutamine metabolism,
365 since proline is a precursor for glutamate and glutamine. We have previously shown that
366 proline, among several other amino acids, differs in women due to habitual diet, i.e. if
367 consuming a vegetarian diet or not [38].

368 4.5 Strengths and limitations

369 The main limitation of the present study is the small sample size. Strengths include the use
370 of both erythrocytes and plasma phospholipids to confirm compliance. Since erythrocyte
371 fatty acids are membrane lipids they reflect more long-term intake in contrast to plasma
372 fatty acids that reflect short-term intake. Also, the cross over design, and applying the OPLS-
373 EP model are strengths since individual factors such as BMI, age and differences in medical
374 treatment do not influence the results to the same extent when comparing the individuals
375 to themselves.

376

377 4.6 Conclusions

378 Our results indicate that modelling fatty acids in erythrocytes is a better biomarker for
379 seafood in dietary interventions, than only EPA and DHA content, although this must be
380 confirmed in other studies. In contrast, ^{1}H-NMR serum metabolomics does not appear to be
381 a good tool to detect markers of blue mussel intake in women with RA. Our data indicate

that factors related to BMI largely influence serum metabolites, which should be considered when comparing groups based on the features observed by metabolomics. Changes in erythrocytes fatty acid pattern could be important for the reduction in disease activity after blue mussel intake in the MIRA-study, though it is possible that other nutrients than n-3 LC PUFAs potentiate the effect, or that unknown factors influenced the results.

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526 Table 1. Models statistics

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Table 1. Models statistics

Model nr	Model	Scaling	Nr of Lv[a]	N	R2X [cum][b]	R2Y [cum][c]	Q2 [cum][d]	CV-ANOVA[e] (p-value)	Permutation test (Q2)[f]	ROC[g] curve (C/M)	Correct classified (%C/%M)	ROC[g] curve test set (C/M)	Correct classified test set (%)
1	**PCA RBC%**	**UV**	3	92	0.449		0.072						
2	**PCA PL%**	**UV**	3	92	0.530		0.168						
3	**PCA metabolites**	**UV**	5	92	0.472		0.212						
4	**OPLS RBC% (y-values: TAG, HDL, Apo A1)**	**UV**	3+0+0	92	0.411	0.508	0.271	< 0.01 all y	TAG -0.137, HDL -0.129, Apo A1 -0.124				
5	**OPLS PL% (y-values: TAG, HDL, Apo A1)**	**UV**	3+0+0	92	0.508	0.549	0.391	< 0.01 all y	TAG -0.142, HDL -0.129, Apo A1 -0.125				
6	**OPLS metabolites (y-values: BMI, TAG, PC)**	**UV**	3+3+0	92	0.473	0.850	0.401	< 0.01 all y	BMI -0.339, TAG -0.364, PC -0.343				
7	**OPLS-EP Δmussels vs Δcontrol RBC%**	**UVN**	1+1+0	23	0.295	0.920	0.715						
8	**OPLS-EP Δmussels vs Δcontrol PL%***	**UVN**	0+0+0	23	0.335	0.539	-0.820						
9	**OPLS-EP Δmussels vs Δcontrol metabolites**	**UVN**	1+0+0	23	0.212	0.423	0.099						
10	**OPLS-DA Δmussels vs Δcontrol RBC%**	**UV**	1+5+0	46	0.599	0.860	0.550	0.00023	-1.06	1.00/1.00	100/100	0.97/0.97	87
11	**OPLS-DA Δmussels vs Δcontrol PL%**	**UV**	1+0+0	46	0.187	0.209	-0.076	1	-0.162	0.76/0.76	70/78	0.64/0.64	59
12	**OPLS-DA Δmussels vs Δcontrol metabolites***	**UV**	1+1+0	46	0.194	0.721	-0.136	1	-0.276	1.00/1.00	91/91	0.58/0.58	50

PCA, principle component analysis; OPLS, Orthogonal Projections to Latent Structures; OPLS-DA, OPLS with Discriminant Analysis, OPLS-EP-OPLS with effect projections, TAG, triacylglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Apo A1,Apolipoprotein A1; BMI, body mass index; PC, platelet count; a Latent Variables, b Cumulative fraction of the sum of squares of X explained by the selected latent variables, c Cumulative fraction of the sum of squares of Y explained by the selected latent variables, d Cumulative fraction of the sum of squares of Y predicted by the selected latent variables, estimated by cross validation, e ANalysis Of VAriance testing of Cross-Validated predictive residuals, f The intercept between real and random models, degree of overfit. g Receiver Operating Characteristics Curve, %C = percentage of control diet sampels, %M= percentage of blue mussel diet samples. * This model was created by forcing the program to build a model with the two first components.

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533 Table 2. Fatty acids (% of total) in erythrocytes and plasma phospholipids before and after the control or mussel diet periods.

		Erythrocytes (%)							Plasma phospholipids (%)						
		Control			Blue mussel				Control			Blue mussel			
Fatty acid		Pre	Post	Δ	Pre	Post	Δ	*p*	Pre	Post	Δ	Pre	Post	Δ	*p*
Myristic acid	14:0	0.31 (0.06)	0.32 (0.06)	0.01 (0.06)	0.31 (0.09)	0.32 (0.06)	0.01 (0.07)		0.99 (0.33)	0.95 (0.26)	-0.04 (0.39)	1.05 (0.39)	0.94 (0.33)	-0.11 (0.37)	
Palmitic acid	16:0	24.49 (0.73)	24.53 (0.75)	0.04 (0.56)	24.47 (0.77)	24.32 (0.64)	-0.15 (0.51)	0.08	23.98 (1.69)	24.11 (1.61)	0.14 (1.8)	24.43 (1.76)	23.8 (1.37)	-0.64 (1.4)	0.073
Stearic acid	18:0	15.87 (0.68)	15.9 (0.72)	0.03 (0.59)	15.92 (0.82)	15.97 (0.61)	0.05 (0.47)		8.27 (0.7)	8.44 (0.82)	0.17 (0.79)	8.46 (0.73)	8.21 (0.55)	-0.25 (0.64)	0.098
Arachidic acid	20:0	0.09 (0.02)	0.09 (0.02)	0 (0.02)	0.09 (0.01)	0.09 (0.01)	0 (0.02)		0.05 (0.01)	0.05 (0.01)	0 (0.01)	0.05 (0.01)	0.05 (0.01)	0 (0.02)	
Behenic acid	22:0	0.06 (0.01)	0.06 (0.01)	0 (0.02)	0.06 (0.01)	0.06 (0.01)	0 (0.02)		0.03 (0.01)	0.03 (0.01)	0 (0.01)	0.03 (0.01)	0.03 (0.01)	0 (0.01)	
Lignoceric acid	24:0	0.26 (0.04)	0.29 (0.06)	0.03 (0.05)	0.28 (0.05)	0.27 (0.04)	-0.01 (0.04)		0.07 (0.02)	0.07 (0.02)	0 (0.02)	0.07 (0.02)	0.06 (0.02)	0 (0.02)	
Σ Saturated fatty acids	SFA	41.08 (0.53)	41.19 (0.46)	0.11 (0.56)	41.12 (0.48)	41.02 (0.46)	-0.1 (0.43)	0.035*	34.08 (2.1)	33.08 (1.4)	0.26 (2.48)	33.39 (2.07)	33.65 (1.91)	-1.01 (1.59)	0.049*
Palmitoleic acid	16:1n-7	0.32 (0.11)	0.34 (0.12)	0.02 (0.07)	0.33 (0.12)	0.31 (0.08)	-0.02 (0.07)	0.043*	2.28 (0.79)	2.18 (0.67)	-0.09 (0.44)	2.26 (0.75)	2.13 (0.56)	-0.13 (0.5)	
Elaidic acid	18:1n-9	16.29 (1.1)	16.19 (1.02)	-0.1 (0.41)	16.18 (0.96)	16.22 (1.11)	0.04 (0.46)		23.26 (3.22)	22.96 (3.49)	-0.3 (1.56)	23 (2.51)	23.01 (2.72)	0.01 (1.45)	
Vaccenic acid	18:1n-7	1.3 (0.16)	1.3 (0.16)	0.01 (0.08)	1.31 (0.18)	1.29 (0.17)	-0.02 (0.07)		1.72 (0.26)	1.74 (0.3)	0.02 (0.26)	1.72 (0.31)	1.78 (0.24)	0.06 (0.18)	
Eicosenoic acid	20:1n-9	0.3 (0.07)	0.29 (0.07)	-0.01 (0.02)	0.3 (0.1)	0.29 (0.07)	-0.01 (0.04)		0.17 (0.04)	0.17 (0.04)	0 (0.03)	0.17 (0.04)	0.18 (0.04)	0.01 (0.05)	
Erucic acid	24:1n-9	0.28 (0.03)	0.28 (0.04)	0 (0.03)	0.27 (0.04)	0.27 (0.04)	0 (0.04)		0.05 (0.02)	0.05 (0.01)	0 (0.02)	0.05 (0.01)	0.05 (0.01)	0 (0.01)	
Σ Mono unsaturated fatty	MUFA	18.49 (1.19)	18.41 (1.11)	-0.08 (0.47)	18.4 (1.06)	18.38 (1.23)	-0.02 (0.48)		27.19 (3.15)	27.14 (3.11)	-0.38 (1.62)	27.49 (3.91)	27.11 (4.08)	-0.05 (1.76)	
Linoleic acid	18:2n-6	11.43 (1.74)	11.34 (1.78)	-0.09 (0.84)	11.42 (1.62)	11.23 (1.47)	-0.19 (0.55)		26.06 (4.72)	25.6 (4.58)	-0.46 (2.12)	25.82 (4.27)	26.13 (3.96)	0.31 (2.58)	
Gamma-linolenic acid	18:3n-6	0.03 (0.01)	0.03 (0.01)	0 (0.01)	0.03 (0.01)	0.03 (0.02)	0 (0.01)		0.3 (0.14)	0.29 (0.1)	-0.01 (0.13)	0.29 (0.13)	0.24 (0.1)	-0.04 (0.09)	
Eicosadienoic acid	20:2n-6	0.21 (0.04)	0.23 (0.04)	0.01 (0.05)	0.22 (0.05)	0.22 (0.08)	0 (0.07)		0.19 (0.05)	0.19 (0.05)	0 (0.05)	0.19 (0.06)	0.19 (0.05)	0 (0.05)	
Dihomo-gamma-linolenic	20:3n-6	1.81 (0.33)	1.86 (0.33)	0.05 (0.18)	1.84 (0.37)	1.74 (0.34)	-0.1 (0.19)	0.002**	1.57 (0.33)	1.62 (0.34)	0.05 (0.33)	1.56 (0.33)	1.55 (0.26)	-0.01 (0.2)	
Arachidonic acid	20:4n-6	15.58 (1.28)	15.97 (1.28)	0.4 (0.83)	15.85 (1.52)	15.56 (1.5)	-0.29 (0.6)	0.005**	6.02 (1.49)	6.73 (1.8)	0.72 (1.08)	6.04 (1.5)	6.34 (1.73)	0.29 (0.78)	
Σ Omega-6	n-6	29.06 (1.66)	29.43 (1.63)	0.37 (0.71)	29.37 (1.57)	28.79 (1.4)	-0.58 (0.65)	0.000053**	33.91 (4.2)	34.45 (3.69)	0.3 (2.32)	34.13 (4.77)	34.43 (4.23)	0.54 (2.59)	
α-Linolenic acid	18:3n-3	0.2 (0.07)	0.19 (0.05)	-0.01 (0.05)	0.2 (0.06)	0.19 (0.06)	0 (0.06)		0.79 (0.24)	0.69 (0.22)	-0.1 (0.2)	0.76 (0.18)	0.74 (0.2)	-0.02 (0.24)	
Eicosatetraenoic acid	20:4n-3	0.1 (0.03)	0.1 (0.03)	-0.01 (0.03)	0.1 (0.03)	0.11 (0.04)	0.01 (0.04)		0.13 (0.07)	0.12 (0.09)	-0.01 (0.06)	0.13 (0.06)	0.11 (0.05)	-0.02 (0.04)	
Eicosapentaenoic acid	20:5n-3	1.45 (0.41)	1.34 (0.39)	-0.11 (0.35)	1.36 (0.32)	1.68 (0.39)	0.31 (0.24)	0.000052**	1.2 (0.57)	1.16 (0.38)	-0.04 (0.56)	1.1 (0.38)	1.4 (0.57)	0.3 (0.47)	0.046*
Docosapentaenoic acid	22:5n-3	3.09 (0.4)	3.1 (0.39)	0.01 (0.25)	3.09 (0.38)	3.11 (0.4)	0.03 (0.14)		0.49 (0.12)	0.5 (0.12)	0.01 (0.08)	0.51 (0.13)	0.53 (0.12)	0.02 (0.08)	
Docosahexaenoic acid	22:6n-3	6.54 (0.79)	6.26 (0.74)	-0.28 (0.46)	6.37 (0.77)	6.72 (0.7)	0.35 (0.36)	0.000010**	2.39 (0.6)	2.35 (0.56)	-0.05 (0.53)	2.32 (0.51)	2.55 (0.52)	0.23 (0.38)	0.072
Σ Omega-3	n-3	11.38 (1.31)	10.98 (1.24)	-0.4 (0.72)	11.12 (1.21)	11.81 (1.17)	0.7 (0.57)	0.000002**	4.82 (0.88)	5.33 (1.09)	-0.18 (1.13)	5 (1.21)	4.81 (0.94)	0.51 (0.85)	0.032*
Σ Poly unsaturated fatty	PUFA	40.43 (1.21)	40.41 (1.27)	-0.03 (0.55)	40.48 (1.2)	40.6 (1.17)	0.12 (0.59)		38.72 (4.39)	39.78 (3.93)	0.12 (2.33)	39.13 (4.84)	39.25 (4.33)	1.06 (2.68)	

Presented values are mean (S.D.), P-values comparing blue mussel diet and control diet are analysed with a mixed model. Only p-values < 0.1 are presented. *<0.05, **<0.01

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Captions to figures

Figure 1 Principal Component Analysis (PCA) for A) Loading scatter plot for erythrocyte fatty acids (%) and B) Loading scatter plot for plasma phospholipid fatty acids (%). (n=92).

Figure 2. A) Model estimation of the projected prediction for all individuals in OPLS-EP models for erythrocyte fatty acids (%). YPred=1 indicates the target value for the model. Each individual corresponds to one observation and the magnitude of the projected effect is given by the height of the corresponding black bar. Deviations from the value 1 for a specific subject indicate a larger or smaller effect on the fatty acid pattern (difference between concentrations in fatty acids after the two diets). B) OPLS-DA models showing separation for erythrocyte fatty acids (%) (model 7, table 1),

Figure 3. Individual data for the omega-3 index over time. Time point 1 is start and 2 the end of the first study period and time point 3 is the start and 4 the end of the second period. The lines are coloured according to what diet the individual started with.

Figure 4. Principal Component Analysis (PCA) for metabolites (n=92, model 1). A) Score scatter plot for component one (17.2% of the explained variation (R2X)). Inverted triangles and stars are samples from individuals with BMI>35 kg/m^2. B) Score scatter plot for component three (13.8% of the explained variation (R2X)). Rhombs are samples from an individual with high white blood cells (>10) and high platelet count (>500).