

The Influence of n-3 Polyunsaturated Fatty Acids and Very Low Calorie Diet during a Short-term Weight Reducing Regimen on Weight Loss and Serum Fatty Acid Composition in Severely Obese Women

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Summary

Polyunsaturated fatty acids of n-3 series (n-3 PUFA) were shown to increase basal fat oxidation in humans. The aim of the study was to compare the effect of n-3 PUFA added to a very low calorie diet (VLCD), with VLCD only during three-week inpatient weight reduction. Twenty severely obese women were randomly assigned to VLCD with n-3 PUFA or with placebo. Fatty acids in serum lipid fractions were quantified by gas chromatography. Differences between the groups were determined using ANOVA. Higher weight (7.55 ± 1.77 vs. 6.07 ± 2.16 kg, NS), BMI (2.82 ± 0.62 vs. 2.22 ± 0.74 , $p < 0.05$) and hip circumference losses (4.8 ± 1.81 vs. 2.5 ± 2.51 cm, $p < 0.05$) were found in the n-3 group as compared to the control group. Significantly higher increase in beta-hydroxybutyrate was found in the n-3 group showing higher ketogenesis and possible higher fatty acid oxidation. The increase in beta-hydroxybutyrate significantly correlated with the increase in serum phospholipid arachidonic acid (20:4n-6; $r = 0.91$, $p < 0.001$). In the n-3 group significantly higher increase was found in n-3 PUFA (eicosapentaenoic acid, 20:5n-3, docosahexaenoic acid, 22:6n-3) in triglycerides and phospholipids. The significant decrease of palmitoleic acid (16:1n-7) and vaccenic acid (18:1n-7) in triglycerides probably reflected lower lipogenesis. A significant negative correlation between BMI change and phospholipid docosahexaenoic acid change was found ($r = -0.595$, $p < 0.008$). The results suggest that long chain n-3 PUFA enhance weight loss in obese females treated by VLCD. Docosahexaenoate (22:6n-3) seems to be the active component.

Key words

Obesity treatment • Fatty acid composition • Very low calorie diet • Beta-hydroxybutyrate • PUFA

Introduction

Caloric restriction and weight loss are responsible for a modification of gene expression in adipose tissue while the proportion of dietary fat does not seem to be decisive (Arvidson *et al.* 2004, Viguerie *et al.* 2005). Composition of dietary fat has been shown to modify the effect of weight reducing diets (Kriketos *et al.* 2001, Clifton *et al.* 2004). Reducing the energy intake tends to dilute the changes in membrane phospholipid fatty acid composition reflecting dietary fat composition in rats (Cha and Jones 2000).

In moderately obese women we have previously found a significant increase of phospholipid palmitic acid (16:0) and significant decrease of linoleic acid (18:2n-6) and dihomo-gamma-linolenic acid (20:3n-6) after very low calorie diet (VLCD) (Kunešová *et al.* 2002a). In humans adipose alpha-linolenic acid was permanently reduced during VLCD (Phinney *et al.* 1990). In rats, linoleic and alpha-linolenic acids (18:3n-3) were shown to be rapidly beta-oxidized even under conditions of extreme dietary linoleate deficiency (Cunnane *et al.* 1998). Moreover, enhanced recycling of carbon from linoleic and alpha-linolenic acid to palmitic acid was suggested (Cunnane *et al.* 2003). The conversion of linoleic and alpha-linolenic acids to long chain polyunsaturated acids was shown to be a quantitatively minor route of utilization in healthy women (McCloy *et al.* 2004). The composition of fatty acids in serum lipids and adipose tissue triglycerides reflects the composition of fatty acids in dietary fats. Fatty acid composition in skeletal muscle triglycerides and phospholipids correlates with parameters of insulin resistance and with the risk of type 2 diabetes (Vessby *et al.* 1994a). Positive association of arachidonic acid (20:4n-6, AA) in serum and muscle phospholipids with insulin sensitivity was shown (Pelikánová *et al.* 1989, Borkman *et al.* 1993). Inverse association between insulin sensitivity and serum dihomo-gamma-linolenic acid (20:3n-6, DHGLA) found by Vessby *et al.* (1994b) and Lovejoy *et al.* (2001) reflects higher activity of delta-5 desaturation of DHGLA to AA (Felton *et al.* 2004). High ratios of linoleic to arachidonic acid concentrations have been observed in subjects with insulin resistance (Berry 2001).

Long chain n-3 PUFA enhance lipid oxidation in healthy humans (Delarue *et al.* 1996, Couet *et al.* 1997). In animals, addition of n-3 fatty acids led to a preferential loss of epididymal fat (Raclot *et al.* 1997) associated with decreased cellularity (Růžičková *et al.* 2004). We have

previously reported a strong genetic influence on the composition of serum and adipose tissue lipids under basal conditions in monozygotic twins (Kunešová *et al.* 2002b), after a short-term weight reduction regimen and after one-year of weight stabilization (Kunešová *et al.* 2002a). After the weight loss the consistent intra-pair resemblances for n-3 fatty acids were found despite dietary stress induced by a very low calorie diet, indicating that the conservation and distribution of this family of essential fatty acids is subject to considerable genetic variance in humans. The resemblances were not influenced by differences in the diet. This was shown when comparing twins concordant and discordant in fat intake.

The present study shows the effect of a short-term inpatient weight-reducing regimen consisting of a very low calorie diet (VLCD) and long chain n-3 PUFA as compared to weight-reducing regimen with VLCD only in severely obese women.

Methods

Subjects

Twenty severely obese women were randomly assigned to a very low calorie diet with n-3 PUFA supplement (n-3 VLCD) or with saline solution (VLCD). There were no significant differences between groups in basal BMI (n-3 VLCD 40.60±4.05; VLCD 45.14±6.9 kg/m²) and age (n-3 VLCD 54.27±5.36; VLCD 49.78±12.35 years). The study was approved by the Charles University Medical Ethical Committee.

Design of the study

The regimen consisted of a one-week eucaloric outpatient baseline stabilization period followed by three weeks of inpatient weight reducing period. During the baseline week and last 3 days of the inpatient stay the subjects did not change their weight. Weight reducing regimen consisted of VLCD Redita^R (Promil Nový Bydžov, Czech Republic) providing 2200kJ/day, comprised of 40 g protein, 70 g carbohydrates and 9 g fat, with addition of n-3 highly unsaturated fatty acids 2.8 g/day (Omega 3 Forte^R, SVUS Pharma, Hradec Kralove, CzR), (n-3 VLCD) or VLCD with saline solution (VLCD group). Supplement with n-3 consisted of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in ratio 2:1, vitamin E 0.9 mg/500 mg capsule was added to prevent fatty acid peroxidation. The reduction regimen included also daily light to moderate

physical activity lasting about 60 min/day. Compliance to the diet was tested by daily semiquantitative measurement of urine ketone bodies and adherence to the physical training by pedometers (Hainer *et al.* 2000). All these procedures were undertaken in the Third Department of Internal Medicine, Charles University in Prague. At the beginning of the weight reducing regimen and on the day 3, 7, and 21 of fasting blood was withdrawn for determination of beta-hydroxybutyrate, free fatty acids, total cholesterol, HDL-cholesterol, triglycerides, fasting blood glucose, fasting insulin, superoxide dismutase, malonyldialdehyde, C and E vitamins. Laboratory analyses were performed by routine laboratory methods. Serum beta-hydroxybutyrate was assessed fluorimetrically (Olsen 1971) and non-esterified free fatty acids were determined photometrically (Duncombe 1964).

At baseline and on day 21, serum lipid fraction of fatty acid analysis was carried out (serum phospholipids – PL, triglycerides – TG and cholesteryl esters – CE); at the same time abdominal adipose tissue was also obtained by needle biopsy. Analysis of the results from adipose tissue samples will be reported elsewhere. Proteins of acute phase fibrinogen, C-reactive protein, α_1 antitrypsin, orosomukoid, α_2 macroglobulin, transferrin and prealbumin were also assessed by routine laboratory methods.

Body composition and regional tissue distribution

Anthropometric estimation of body fat was performed by measurement of ten skinfolds according to Pařízková (1977) and of four skinfolds according to Durnin and Wommersley (1974). Waist and hip circumference and sagittal abdominal diameter at the level L4/5 were measured following the standardized procedure recommended at the Airlie Conference (Lohman *et al.* 1989). Body fat content was estimated by bioelectrical impedance measurement (Tanita TBF 105, Japan).

Fatty acid composition

Fatty acid composition of serum lipids was performed by gas chromatography after separation of individual serum fractions by thin-layer chromatography on silica gel. Detailed description of the method is given elsewhere (Tvrzická *et al.* 2002).

Statistical methods

Relationships between one dependent variable

and the set of independent variables were evaluated using a stepwise backward multiple regression. Prior to the analysis, the original dependent variables were transformed by power transformation using a normal probability plot for finding the best transformations. The minimum value of the mean squared error of the linear regression fit between theoretical fractiles of the Gaussian distribution and experimental fractiles indicated the optimum transformation parameter. Severe non-homogeneities as detected using the aforementioned plot were not included in the finding of the optimum transformation parameters. Nevertheless, these outliers were included in further processing. The optimum transformation of the dependent variable was searched using the minimum skewness of Studentized residuals of the multiple regression model as an indicator. Further regression diagnostics was performed as described elsewhere (Meloun *et al.* 2002, 2004). The relationships between two variables were estimated using Pearson's correlations. Respecting a non-Gaussian distribution and a non-constant variance in some variables, these were transformed prior to testing as described above. For transformation strategy see also our previous study (Meloun *et al.* 2000). The effects of status (patients, controls) and stage of the treatment (before treatment, after treatment) and a between-factor interaction were evaluated using a repeated measures ANOVA model. Again, respecting the non-Gaussian data distribution in some variables, such data underwent power transformations. The transformation strategy as well as the residual analysis was analogous as in the regression.

The results tested by the aforesaid ANOVA model were checked by non-parametric tests of the differences between the beginning and end of the treatment. The significances of the differences were evaluated using Wilcoxon's paired test. To estimate how the differences diverged between controls and patients, the Mann-Whitney test of their means was applied.

Data are expressed as means \pm S.E.M. $P=0.05$ was taken as the threshold of statistical significance.

Results

Baseline characteristics of the subjects before the treatment and the effect of the treatment are presented in Table 1. Weight loss (7.55 ± 1.77 vs. 6.07 ± 2.16 kg, $p<0.10$) and BMI decrease (2.82 ± 0.62 vs. 2.22 ± 0.74 kg/m², $p<0.05$) were higher in the n-3 PUFA supplemented group. The decrease of hip circumference

was significantly higher after n-3 VLCD (4.8 ± 1.81 cm) than in the VLCD (2.5 ± 2.51 cm, $p < 0.05$) group. Higher increase in beta-hydroxybutyrate was found in n-3 group

($p < 0.01$, Fig. 1A). A tendency to higher decrease in fibrinogen was observed in n-3 group ($p < 0.09$, Fig. 1B).

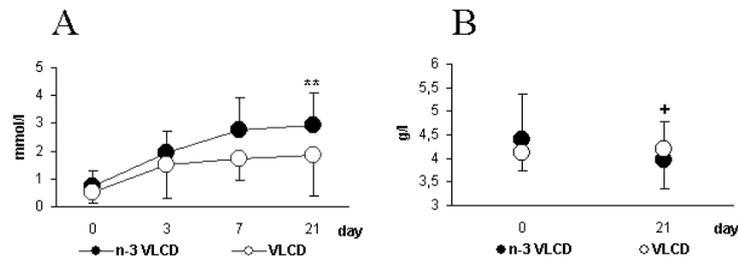


Fig. 1. **A.** Change in serum beta-hydroxybutyrate during the weight-reducing regimen ** $p < 0.01$. **B.** Change in serum fibrinogen during the weight-reducing regimen + NS ($p < 0.09$), Values are expressed as mean \pm S.D.

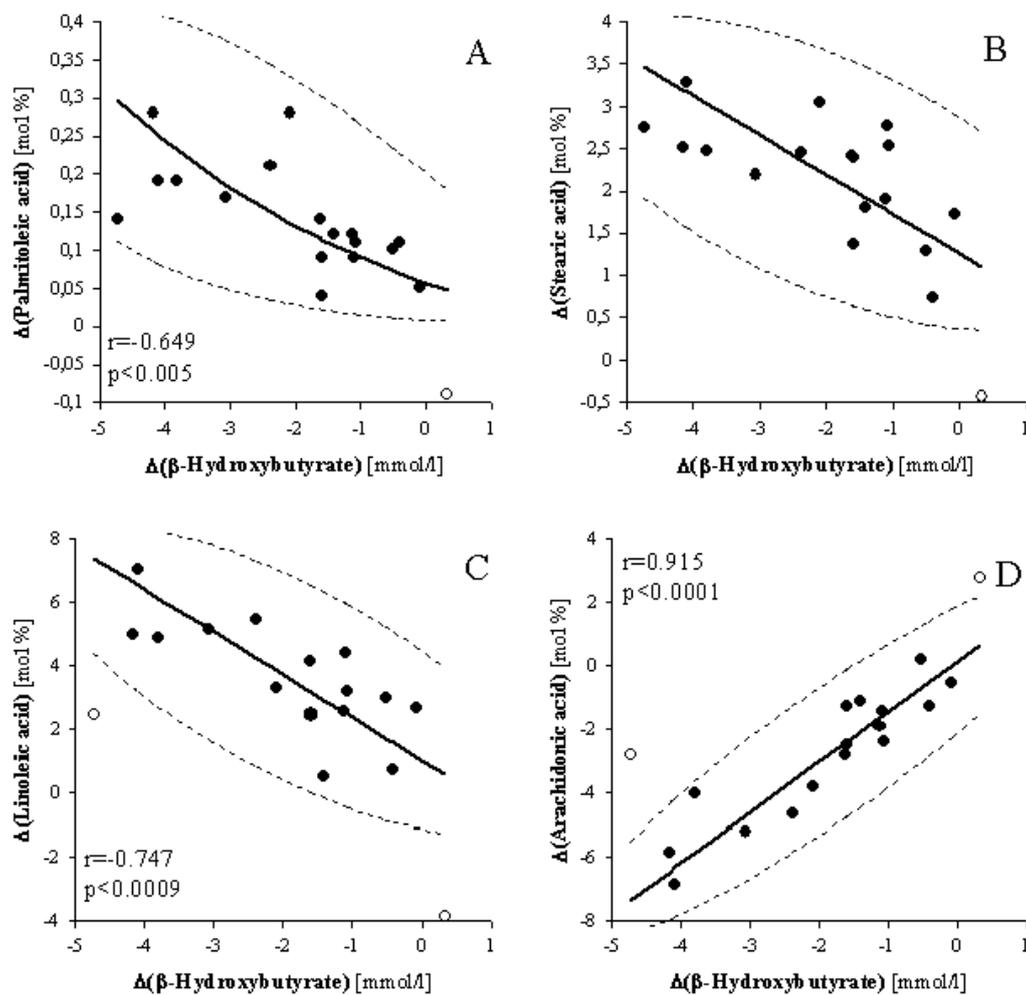


Fig. 2. Correlation of the change in beta-hydroxybutyrate and change in palmitoleic acid (16:1n-7) **(A)**, change in stearic acid (18:0) **(B)**, change in linoleic acid (18:2n-6) **(C)**, change in arachidonic acid (20:4n-6) **(D)**. Pearson's correlations between the change of beta-hydroxybutyrate and change in proportion of individual fatty acids in serum phospholipids. The full and empty circles indicate the individual subjects included in the analysis and outliers, respectively. The full and dashed curves indicate the retransformed principal axes and their 95 % confidence ellipsoids (the area in which the 95 % of the experimental points should be theoretically found provided that no outliers are present), respectively. Respecting the non-Gaussian data distribution and the non-constant variance the data were transformed using a power transformation prior analysis (for details see Statistical data treatment). The symbols r and p represent the correlation coefficients and their statistical significances when correlating the transformed data.

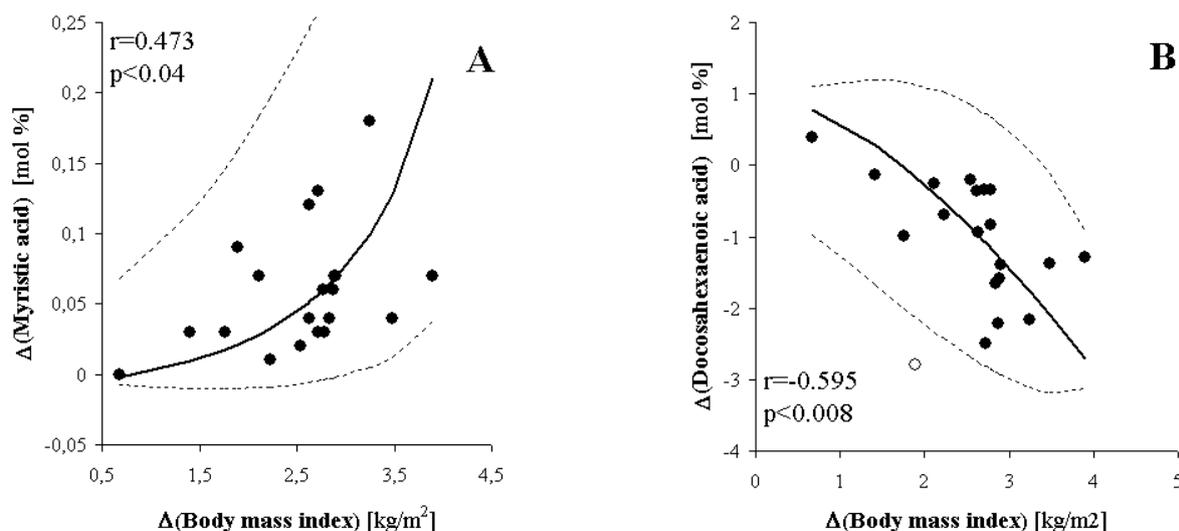


Fig. 3. Pearson's correlations between the change of BMI and change in proportion of myristic acid (14:0) (A) and docosahexaenoic acid (20:6n-3) (B). For comments see Fig. 2.

Table 1. Characteristics of the group before the treatment and the effect of the weight-reducing regimen

Variable	N3 VLCD (n=11)	VLCD (n=9)
<i>Initial values</i>		
Age (years)	54.27±5.36	49.78 ± 12.35
Weight (kg)	108.2±8.7	123.2 ±23.7
BMI (kg/m ²)	40.60 ±4.05	45.14 ±6.9
Waist (cm)	116.2±10.0	119.4±11.6
Hip (cm)	136.0±7.8	140.4±13.1
<i>Effect of treatment</i>		
Weight loss (kg)	7.55±1.77	6.07±2.16 **
BMI loss (kg/m ²)	2.82±0.62	2.22±0.74 *
Waist (cm)	5.5±1.71	3.3±3.41
Hip (cm)	4.8±1.81	2.5±2.51 *

* p<0.05 ** NS (p<0.1), Values are expressed as mean ± S.D.

Table 2 shows the composition of fatty acids in serum phospholipids (PL) and triglycerides (TG) before treatment and after the treatment in the n-3 VLCD and VLCD groups. An increase in eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acids (DHA, 22:6n-3) in both PL and TG was found. In TG a significantly higher decrease of palmitoleic (16:1n-7, POA) and vaccenic (18:1n-7) acids and significantly higher increase of oleic acid (18:1n-9) was observed. Palmitoleate was shown as a marker of lipogenesis previously (Fukuda *et al.* 1999, Kunešová *et al.* 2002b). A highly significant negative

correlation between changes in levels of beta-hydroxybutyrate and in serum POA proportions in PL (p = -0.83, p<0.0001), TG and CE (r = -0.69, p<0.005, r = -0.49, p<0.05) respectively, could indicate a higher level of hepatic beta-oxidation. However, it could also suggest a lower lipogenesis. Negative correlation between initial BMI and change in POA was found (r = -0.45, p<0.05).

A higher decrease of TG POA was shown in the n-3 VLCD treated group. A higher hepatic beta-oxidation in response to n-3 supplement could be inferred by a higher increase of beta-hydroxybutyrate in n-3 treated group. Significantly higher increase in TG oleic acid (18:1n-9, OA) in n-3 VLCD was shown. A strong positive correlation was observed between the change of PL, TG and CE arachidonic acid and the change of beta-hydroxybutyrate (r = 0.92, p<0.001; r = 0.68, p<0.005; r = 0.66, p<0.005, respectively); significant negative correlation of beta-hydroxybutyrate change with change of palmitic (16:0), stearic (18:0) and linoleic (18:2n-6) acids in phospholipids was also shown (Fig. 2). The increase in PL DHA correlated significantly with BMI decrease (r = -0.595, p = 0.008) and there was a positive correlation of myristate change (14:0) with BMI change (Fig. 3). Serum triglyceride change correlated positively with TG palmitate change and a negative correlation with TG docosahexaenoic acid (22:6n-3) change was found (data not shown). The changes in serum triglycerides, serum non-esterified fatty acids and fasting blood glucose levels were not significantly different between n-3 VLCD and VLCD groups (data not shown).

Table 2. Fatty acid composition in serum lipids before and after the treatment

Phospholipids Fatty acid	n-3 VLCD (n=11)		VLCD (n=9)	
	Baseline	Day 21	Baseline	Day 21
12:0	0.01±0.002	0.009±0.002	0.004±0.002	0.004±0.002
14:0	0.21±0.02	0.13±0.002	0.16±0.009	0.122±0.009
16:0	31.71±0.34	34.79±0.30	31.16±0.51	33.73±0.73
16:1n-9	0.14±0.008	0.19±0.008	0.14±0.007	0.13±0.001
16:1n-7	0.68±0.03	0.53±0.04	0.57±0.03	0.49±0.04
18:0	12.57±0.38	10.29±0.32	13.18±0.34	11.23±0.53
18:1n-9	8.86±0.31	8.71±0.38	9.22±0.33	9.48±0.18
18:1n-7	1.57±0.07	1.73±0.05	1.68±0.05	1.84±0.005
18:2n-6	20.86±0.05	16.68±0.73	20.11±0.57	18.67±0.69
18:3n-6	0.06±0.005	0.03±0.003	0.05±0.004	0.04±0.007
18:3n-3	0.13±0.008	0.09±0.005	0.12±0.008	0.10±0.011
20:0	0.04±0.002	0.03±0.009	0.03±0.002	0.03±0.001
20:1	0.11±0.007	0.11±0.006	0.12±0.006	0.12±0.006
20:2n-6	0.28±0.02	0.18±0.008	0.26±0.02	0.20±0.01
20:3n-6	3.67±0.22	1.95±0.06	3.73±0.20	2.1. ±0.17
20:4n-6	12.46±0.47	15.68±0.94	13.58±0.74	15.18±1.01
20:5n-3	1.05±0.10	1.68±0.15***	0.77±0.05	0.63±0.03
22:5n-3	0.925±0.06	1.04±0.07	0.88±0.04	0.904±0.04
22:6n-3	4.17±0.27	5.76±0.22*	4.07±0.21	4.52±0.27

Triglycerides Fatty acid	n-3 VLCD (n = 11)		VLCD (n = 9)	
	Baseline	Day 21	Baseline	Day 21
12:0	0.08±0.01	0.04±0.004	0.05±0.007	0.03±0.006
14:0	1.25±0.08	0.72±0.05	0.97±0.06	0.60±0.07
16:0	28.46±0.14	27.35±0.73	26.57±0.31	26.17±0.38
16:1n-9	0.76±0.04	0.61±0.04	0.72±0.03	0.56±0.04
16:1n-7	4.33±0.12	3.06±0.20*	3.64±0.21	3.06±0.28
18:0	2.59±0.07	2.33±0.09	2.51±0.19	2.08±0.11
18:1n-9	38.93±0.79	39.88±0.42*	41.39±0.49	41.84±0.79
18:1n-7	2.63±0.06	2.54±0.84*	2.86±0.07	2.79±0.09
18:2n-6	16.24±0.75	17.14±0.82	16.39±0.51	17.19±0.82
18:3n-6	0.18±0.04	0.15±0.01	0.19±0.01	0.17±0.02
18:3n-3	0.73±0.05	0.69±0.05	0.83±0.34	0.75±0.03
20:0	0.02±0.001	0.02±0.002	0.02±0.007	0.02±0.005
20:1	0.18±0.008	0.14±0.003	0.19±0.01	0.17±0.01
20:2n-6	0.12±0.007	0.09±0.004	0.11±0.005	0.09±0.004
20:3n-6	0.34±0.05	0.20±0.001	0.29±0.02	0.20±0.02
20:4n-6	1.58±0.21	2.13±0.23	1.60±0.12	2.10±0.18
20:5n-3	0.21±0.04	0.49±0.07**	0.17±0.01	0.19±0.03
22:5n-3	0.37±0.03	0.52±0.04	0.38±0.03	0.47±0.03
22:6n-3	0.76±0.08	1.22±0.10 [#]	0.72±0.06	0.93±0.10

[#] p<0.1 *p<0.05 **p<0.01 in comparison with change in the control group, Values are expressed as means ±S.E.M. (in mol %)

Discussion

The main finding of the study is that in severely obese females, n-3 PUFA supplementation added to VLCD significantly enhanced BMI loss and reduction of hip circumference. The secondary finding is a significantly higher elevation of serum beta-hydroxybutyrate in n-3 PUFA supplemented group. Both of these effects occurred despite the fact that the baseline mean BMI for the subjects in the n-3 VLCD group tended to be lower than that for the saline VLCD group, whereas one would expect to observe greater weight loss and higher ketones in heavier subjects given a fixed energy of VLCD.

Similar increase in ketogenesis was found in epileptic patients after using polyunsaturated fatty acids instead of saturated fats during ketogenic diet which lead to a higher increase in beta-hydroxybutyrate (and also in insulin sensitivity) (Fuehrlein *et al.* 2004). In the above study, the composition of PUFA in the diet was not given. A higher ketosis after diet containing n-3 PUFA in comparison with saturated fats is consistent with the results found in animals (Storlien *et al.* 1987, Likhodii *et al.* 2000). In epileptic children treated by ketogenic diet arachidonate and docosahexaenoate in plasma free fatty acids increased simultaneously with beta-hydroxybutyrate elevation (Fraser *et al.* 2003). The increase in ketogenesis is probably caused by a higher fatty acid oxidation after n-3 PUFA supplementation. In rats, higher beta-oxidation was shown as a mediator of the hypotriglyceridemic effect of the n-3 PUFA (Ukropec *et al.* 2003). Stimulation of fatty acid oxidation in the liver is caused by the activation of peroxisome proliferator activated receptor alpha (Delarue *et al.* 2004)

Significant correlation of beta-hydroxybutyrate change with a change in arachidonate content in all serum lipid classes, especially in phospholipids, was demonstrated for the first time. If there is a causal relationship or simultaneous association should be studied further. Compliance to the supplementation was high as indicated by the increase in proportion of EPA and DHA in serum phospholipids and triglycerides.

The enhanced level of arachidonic acid after both treatments could be caused by its higher release from adipose tissue. On the other hand, a lower metabolism of AA after fasting and refeeding was shown by Qu *et al.* (1998), who demonstrated significant reduction in total hepatic microsomal AA metabolism in

rat liver, concurrently with weight loss and increased beta-hydroxybutyrate levels. In addition to cyclooxygenases and lipoxygenases, cytochrome P450 monooxygenases metabolize AA to compounds which have an important role in the regulation of cellular processes. Fasting has been reported to increase AA accumulation in hepatic neutral and phospholipid pools (Larsson-Backstrom *et al.* 1990). In humans, Phinney *et al.* (1991) found that arachidonate increased during VLCD, and decreased after finishing the ketogenic diet. Concurrently with AA increase fibrinogen decrease tended to be higher in n-3 VLCD group confirming the previously shown antiinflammatory effect of n-3 PUFA (Browning 2003).

Polyunsaturated fatty acids regulate lipogenic gene expression in different tissues. Clarke and Jump (1994) have demonstrated that dietary fat composition directly affects fatty acid synthase gene expression. As described in rats, a higher decrease of palmitoleic acid in serum triglycerides confirms indirectly the suppression of lipogenic genes including fatty acid synthase (Fukuda *et al.* 1999).

Previous studies in rodents indicated that n-3 PUFA enriched diets prevented accumulation of fat in the abdomen (Raclot *et al.* 1997, Růžičková *et al.* 2004). The effect of n-3 PUFA (EPA, DHA and mixed fat) on lipogenic genes expression was shown in retroperitoneal fat of rats (Raclot *et al.* 1997). The replacement of 3 % (wt/wt) of obesity-promoting HF composite diet with EPA/DHA reduced weight gain and reduced the accumulation of epididymal, but not of subcutaneous fat in mice, simultaneously with a depression of tissue cellularity and favorable changes in glucose homeostasis gene activity (Růžičková *et al.* 2004). The influence of the type of dietary fat on the composition of phospholipids in cellular membrane and possibly also function becomes increasingly important with positive energy balance (Cha and Jones 2000). The effect of n-3 PUFA addition to the diet of obese subjects could be more expressed under conditions of lower energy deficit.

Conclusion

The addition of n-3 PUFA of fish origin to a very low calorie diet results in a greater BMI loss and hip circumference reduction in severely obese women during an inpatient short-term weight-reducing regimen. Higher increase in beta-hydroxybutyrate was shown in the n-3 group probably due to a higher beta-oxidation of fatty

acids. Significant correlation of BMI loss with change in phospholipid docosahexaenoic acid level suggests a causal relationship.

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