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RESEARCH ARTICLES

N-3 fatty acid supplementation decreases plasma homocysteine in diabetic dyslipidemia treated with statin–fibrate combination

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Abstract

The aim of this study was to study the effect of adding polyunsaturated fatty acid (PUFA) n-3 or placebo (containing oleic acid) to a combined statin–fibrate treatment on plasma lipoproteins, lipoperoxidation, glucose homeostasis, total homocysteine (tHcy) and microalbuminuria (MA) in patients with diabetic dyslipidemia (DDL). Twenty-four patients, who did not fulfill the recommended target lipid values with combined hypolipidemic therapy (pravastatin 20 mg+micronized fenofibrate 200 mg daily), were supplemented with 3.6 g PUFA n-3 daily for 3 months or placebo (olive oil) for the next 3 months. The concentrations of plasma lipids, fatty acid (FA) profiles of phosphatidylcholine (PC), cholesteryl esters (CE) and triglycerides (TG), tHcy levels, concentrations of conjugated dienes (CD) in low-density lipoprotein (LDL), and MA were determined in baseline state, after the PUFA n-3 and placebo treatment period. Supplementation with PUFA n-3 led to a significant decrease in plasma tHcy (−29%, $P<.01$) and TG (−28%, $P<.05$) levels, as well as to a significant decrease in MA (−24%, $P<.05$). The decrease in MA correlated significantly with the increase in total PUFA n-3 ($r=-.509$, $P\leq.05$) and docosahexaenoic acid ($r=-.52$, $P<.01$) in TG. The concentrations of CD in LDL increased significantly (+15%, $P<.05$). The supplementation with PUFA n-3 to the combined statin–fibrate treatment in patients with DDL decreased the TG and tHcy levels as well as MA. It could lead to decreased risk of atherothrombosis and delay of diabetic nephropathy onset and progression.
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Keywords: PUFA n-3; Diabetic dyslipidemia; Statin; Fibrate; Total homocysteine; Microalbuminuria

1. Introduction

In the treatment of diabetic dyslipidemia (DDL), the recommended target values of plasma lipids [1] are rarely achieved by monotherapy, and the use of a combined hypolipidemic therapy also often remains without desired results [2,3]. Another possibility to favourably influence DDL is supplementation with polyunsaturated fatty acid (PUFA) n-3, with eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids being the main representatives. This supplementation lowers the plasma TG concentrations (both fasting and postprandial), sometimes increases the low-density lipoprotein cholesterol (HDL-C) concentrations and enlarges the mean particle size of low-density lipoprotein (LDL) [4,5].

Numerous epidemiological studies have proved an association between the content of PUFA n-3 in the diet and a decrease in cardiovascular and overall mortality [6,7].

Polyunsaturated fatty acid n-3 exhibits many beneficial pleiotropic effects (hypolipidemic, antithrombotic, antiarrhytmic and anti-inflammatory); it is also known to restore endothelial dysfunction (ED) [8,9]. A mild hyperhomocysteinemia (hHcy) is considered to be an independent risk factor (RF) for atherothrombosis [10]. The effects of PUFA n-3 supplementation on homocysteine (Hcy) metabolism were not consistently studied. Only a few papers have described a decrease in tHcy concentrations after PUFA n-3 supplementation [11,12].

In nondiabetics, increased plasma tHcy is connected with the development of microalbuminuria (MA), independently of glomerular filtration [13]. Mild hHcy and MA are conventional RFs in diabetics, in whom both RFs

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substantially increase the cardiovascular morbidity [14]; therefore, lowering of plasma Hcy with PUFA n-3 administration could be very useful. Patients with diabetes mellitus (DM) type 2 are often treated with metformin and fibrates. Both drugs are known to elevate the plasma tHcy levels [15,16].

The aim of this study was to determine the influence of PUFA n-3 administration on diabetes control, lipid concentrations, oxidative stress, plasma tHcy levels and MA in severe DDL, so far insufficiently treated with the combination of statin–fibrate.

2. Methods and materials

2.1. Patients

Twenty-four persons (13 males, 11 females) with DM type 2 with severe mixed dyslipidemia were recruited from outpatients of the Fourth Internal Department of the First Faculty of Medicine of Charles University and The General Teaching Hospital, Prague. The patients were treated for at least 12 months with a statin–fibrate combination [pravastatin (Lipostat, Bristol-Myers-Squibb) 20 mg+micronised fenofibrate (Lipanthyl 200 M, 200 mg, Laboratoires Fournier, France) daily] without reaching the recommended target values of plasma lipids [1].

The average age was 48.8 (range 35–64 years); body mass index (BMI) was 29.8±2.9 (mean±S.E.M.). Body mass index was calculated as body weight (in kg) divided by height (in m) squared. Twenty patients were hypertonics; eight were smokers. All patients received instructions for their diet according to the standard protocol for diabetics with dyslipidemia for at least 12 months before inclusion [17]. Weight and BMI were stable for at least 3 months prior to the enrolment in the study. Similarly, glycated haemoglobin and glycaemia were stabilized for two consecutive tests 3 months apart, prior to the inclusion in the study. Patients with signs of liver or kidney disease (creatinine >130 μmol/L), hypothyroidism, malignancies, macroalbuminuria (proteinuria higher than 300 mg/day) and with manifest microvascular or macrovascular complications of DM were excluded from the study. No patient received pyridoxine, cyanocobalamin or folic acid supplementation. All the patients were treated with metformin (a mean daily dose of 1500 mg); the average duration of DM was 6.2 years (2–19 years). All patients signed an informed consent for inclusion in the study. The study was approved by the local ethics committee.

2.2. Study design

The presented study was designed as a single-blind, placebo-controlled study. After being included into the study, the patients were treated during two consecutive 3-month periods. In the first period, they were supplemented with PUFA n-3 in a daily dose of 3.6 g (Omega-3 Forte, SVUS-Pharma, Hradec Králové, Czech Republic); in the second period, with placebo containing 3.6 g of olive oil. The content of EPA and DHA in the Omega-3 Forte preparation was 57.4% and 28.7% (w/w), respectively. The main fatty acids (FAs) in the placebo preparation were oleic (74.9%), palmitic (10.1%) and linoleic (9.2%, w/w) acids.

2.3. Laboratory procedures

At the beginning and end of each period, the following measurements of total cholesterol (TC), TG, HDL-C, nonesterified fatty acids (NEFA), apolipoproteins (apo) A-I and B in plasma, composition of FA in plasma PC, TG and CE, and CD concentrations in LDL were carried out [18].

Blood samples were collected after overnight fasting. The concentrations of TC, TG, uric acid and glucose were assessed by enzymatic–colorimetric methods; HDL-C concentration in supernatant after precipitation of lipoprotein-B with PTA/Mg^{2+}. Non-HDL-C concentration was calculated as the difference between TC and HDL-C concentration. The concentrations of apo were measured by Laurell rocket electroimmunoassay, using standard and specific antibodies [apo B, apo A-I (Behring Werke, Marburg, Germany), Lp[a] (Immuno, Wien, Austria)]. Glycated haemoglobin (HbA_{1c}) was measured by high-performance liquid chromatography (HPLC) (BioRad, Richmond, CA, USA). The concentrations of NEFA were

Table 1
Effects of adding PUFA n-3 supplement (3.6 g/day) to pravastatin+fenofibrate treatment in patients with diabetic dyslipidemia

Parameter	Baseline	PUFA n-3	Placebo	P^a
Total plasma cholesterol (mmol/L)	5.99±0.42[b]	5.69±0.39	5.90±0.45	NS
Triglycerides (mmol/L)	4.77±0.98[c]	3.45±0.67	4.14±0.92[d]	.05
Non-HDL cholesterol (mmol/L)	4.91±0.45	4.70±0.38	4.93±0.46	NS
NEFA (mmol/L)	0.80±0.06	0.88±0.07	0.87±0.11	NS
Apo A-I (g/L)	1.65±0.08	1.70±0.07	1.72±0.07	NS
Apo B (g/L)	1.14±0.07	1.08±0.09	1.11±0.06	NS
Lipoprotein [a] (g/L)	0.21±0.09	0.20±0.09	0.18±0.06	NS
f-Glucose (mmol/L)	9.4±0.9	9.9±0.7	9.7±0.8	NS
HbA_{1c} (%)	7.75±1.94	7.53±0.69	7.56±1.74	NS
Uricaemia (μmol/L)	316±22	309±24	321±23	NS
Homocysteine (μmol/L)	13.78±1.78[c]	9.75±0.84	14.18±1.40[d]	.01
Cyanocobalamin (ng/L)	365±29	385±36	377±38	NS
Folate (g/L)	12.7±3.4	14.6±6.3	13.8±7.7	NS
Microalbuminuria (mg/L)	27.0±8.4	20.5±3.2	29.5±6.1	.05
Albumin/creatinine (g/mol)	6.0±6.7	4.8±2.8	6.3±4.6	NS
Conjugated dienes (μmol/L)	54.2±3.7	62.5±7.8	51.7±6.5[d]	.05

[a] ANOVA.
[b] Mean±S.E.M.
[c] Statistically significant difference between baseline and PUFA n-3: $P<.05$ (Scheffé test).
[d] Statistically significant difference between PUFA n-3 and placebo: $P<.05$ (Scheffé test).

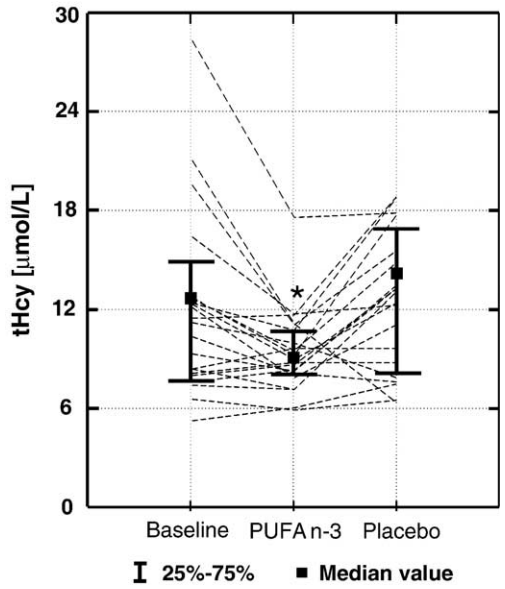

Fig. 1. Effect of PUFA n-3 on plasma tHcy concentrations in 24 patients with severe DDL treated with statin–fibrate combination therapy. Values are given as median and interquartile range (µmol/L). *P<.05, Scheffé's ANOVA post hoc test. Values obtained after PUFA n-3 period were statistically significantly different both from baseline and placebo periods. Dashed lines represent individual cases.

determined by using the enzymatic–colorimetric method (NEFA, Randox Laboratories, UK). The composition of FA in plasma PC, TG and CE was determined by capillary gas chromatography [19]. Serum tHcy concentrations were determined by HPLC according to Araki and Sako [20]. Folic acid and cyanocobalamin concentrations were assessed with a chemiluminiscent assay (ARC folate and B_{12} reagent kits, respectively; Abbott, GB). Microalbuminuria was analyzed by laser nephelometry method (Image MA reagent kit, Beckman Coulter, USA).

Statistical evaluation was performed by means of the Statistica software (Tulsa, OK, USA, 2000).

3. Results

No significant changes in body weight or blood pressure were observed throughout the study. Changes in the concentrations of plasma lipids, apo, NEFA, CD, glucose homeostasis indices, uricaemia, tHcy and MA are shown in Table 1.

Addition of PUFA n-3 in a daily dose of 3.6 g to the combined present therapy resulted in a decrease in plasma TG (−28%, P<.05). The concentrations of apo B-100, apo A-I, Lp[a], NEFA, fasting glycaemia, glycated haemoglobin, uric acid, cyanocobalamin and folic acid did not significantly change. After PUFA n-3 supplementation, there was a statistically significant decrease in plasma tHcy concentration (−29%, P<.05), as shown in Fig. 1, and MA, expressed as milligrams per liter in the morning urine sample (−24%, P<.05). However, when MA was expressed as the ratio albumin/creatinine (g/mol), the changes (−20%)

Table 2
Composition of relevant fatty acid in plasma lipid classes

Fatty acid[1]	Cholesteryl esters			Triglycerides			Phosphatidylcholine		
	Baseline	PUFAn-3	Placebo	Baseline	PUFAn-3	Placebo	Baseline	PUFAn-3	Placebo
16:0	11.5±0.4	11.6±0.3	10.7±0.2	30.8±0.7	30.7±0.7	29.0±0.7	32.0±0.6	32.1±0.6	30.7±0.3
16:1n-7	5.1±2.9	4.8±3.4	4.3±2.4	4.5±2.2	4.5±2.2	4.2±2.0	0.8±0.3	0.7±0.3	0.7±0.4
18:0	0.8±0.9	0.7±0.2	0.7±0.2	3.8±1.4	3.5±1.2	3.7±1.1	14.5±1.4	14.6±1.8	14.1±1.1
18:1n-9	21.1±2.9	20.3±2.8	22.4±3.8	38.6±3.9	37.3±2.7	40.9±2.9	11.0±2.3^{aaa}	9.7±1.8***	11.3±2.2^{bbb}
18:2n-6	45.7±6.7	45.5±7.1	47.1±5.9	12.5±4.1	13.0±3.3	12.7±3.4	18.2±0.5^{a}	16.7±3.7***	19.0±3.7^{bb}
18:3n-6	1.4±0.6^{aaa}	1.1±0.3***	1.2±0.3	0.3±0.1	0.3±0.1	0.3±0.1	0.1±0.05	0.1±0.05	0.1±0.03
18:3n-3	0.5±0.6	0.5±0.1	0.5±0.1	0.8±0.3	0.8±0.2	0.7±0.2	0.2±0.05	0.1±0.05	0.2±0.05
20:3n-6	0.6±0.1	0.6±0.1	0.7±0.1	0.2±0.1	0.2±0.1	0.3±0.1	3.1±0.7^{aaa}	2.6±0.5***	3.1±0.6^{bbb}
20:4n-6	8.4±2.2	7.7±1.3	8.4±2.1	1.5±0.5	1.4±0.4	1.5±0.4	11.9±1.7^{aaa}	10.3±1.3***	12.3±2.1^{bbb}
20:5n-3	1.6±0.6^{aaa}	3.9±1.5***	1.1±0.5^{bbb}	0.4±0.2^{aaa}	1.0±0.5***	0.3±0.1^{bbb}	1.4±0.8^{aaa}	4.1±1.5***	1.2±0.5^{bbb}
22:5n-3	0.04±0.02	0.05±0.01	0.05±0.01	0.3±0.1^{aaa}	0.6±0.2***	0.3±0.1^{bbb}	0.9±0.2^{aaa}	1.1±0.3***	0.8±0.2^{bbb}
22:6n-3	0.5±0.1^{aaa}	0.8±0.2***	0.5±0.2^{bbb}	0.6±0.2^{aaa}	1.6±0.8***	0.7±0.5^{bbb}	3.2±0.6^{aaa}	5.3±1.2***	3.7±0.9^{bbb}
Σ SFA	13.0±1.7^{cc}	13.0±1.3	12.0±0.8	36.4±4.3	35.8±3.9	34.4±3.9	46.4±2.8	47.0±2.9*	45.2±1.1^{b}
Σ MFA	27.9±5.5	26.1±5.9	28.3±5.9	46.6±5.1	45.1±3.9**	48.4±3.1^{bb}	13.4±0.3^{aaa}	12.1±2.3***	13.7±2.6^{bbb}
Σ n-6 PUFA	56.5±6.4	55.1±6.2*	57.6±5.9^{b}	14.9±4.2	15.1±3.4	15.2±3.9	34.1±0.4^{aaa}	30.3±3.5***	35.3±3.2^{bbb}
Σ n-3 PUFA	2.6±0.8^{aaa}	5.2±2.0***	2.2±0.7^{bbb}	2.2±0.6^{aaa}	4.0±1.4***	2.0±0.8^{bbb}	5.7±1.4^{aaa}	10.6±2.5***	5.9±1.3^{bbb}

[1] Mean±S.E.M. (mol%).
[2] ANOVA: *P<.05, **P<.01, ***P<.001.
Statistically significant difference between baseline and PUFA n-3: ^{a}P<.05, ^{aaa}P<.001 (Scheffé test).
Statistically significant difference between PUFA n-3 and placebo: ^{b}P<.05, ^{bb}P<.01, ^{bbb}P<.001 (Scheffé test).
Statistically significant difference between baseline and placebo: ^{c}P<.05, ^{cc}P<.01, ^{ccc}P<.001 (Scheffé test).

did not reach a statistical significance ($P=.38$). When PUFA n-3 administration was replaced by the placebo FA mixture, plasma tHcy and TG increased significantly (+45%, $P<.05$ and +20%, $P<.05$, respectively) close to the starting values as did also the MA (+43%, $P<.05$). Addition of PUFA n-3 led to increased concentrations of CD in LDL (+15%, $P<.05$).

The changes in relevant FA in plasma PC, CE and TG are given in Table 2. Treatment with PUFA n-3 led to a highly significant increase in both total and individual PUFA n-3 in plasma CE, TG and PC, which returned to basal values after placebo administration ($P<.0001$). The supplementation with PUFA n-3 led to a decrease in total PUFA n-6 in CE ($P<.05$) and PC ($P<.0001$). The decrease in FA 18:2 n-6, 20:3 n-6 and 20:4 n-6 (all $P<.0001$) was also observed in PC as well as in total MFA ($P<.001$), and oleic acid (18:1 n-9) ($P<.001$). Administration of the placebo (rich in oleic acid C18:1 n-9) led to a significant increase in total monoenoic FA (MFA) in TG in comparison with PUFA n-3 supplementation and also to a decrease in total saturated FA (SFA) in CE in comparison with the baseline state ($P<.01$).

We found only the following significant correlations (expressed as Pearson's correlation coefficients) between changes in relevant FA and changes in these parameters: tHcy correlated positively with the contents of both stearic acid and SFA in PC ($r=.446$, $P<.05$, $r=.556$, $P<.01$, respectively); MA correlated negatively with the contents of DHA and total PUFA n-3 in TG ($r=-.52$, $P<.05$, $r=-.509$, $P<.05$ respectively) and positively with MFA in CE ($r=.465$, $P<.05$).

4. Discussion

The most important finding of this study was the significant decrease in tHcy and MA after PUFA n-3 supplementation in patients with DDL, who were treated with statin–fibrate combination. The additive hypolipidemic action that was reversed by replacing with placebo (oleic acid) was another important effect of PUFA n-3 supplementation.

According to our knowledge, the addition of PUFA n-3 to a combined statin–fibrate hypolipidemic treatment in DDL was used for the first time in this pilot study. The treatment was well tolerated and without any need of discontinuation. Only several studies reported on the use of PUFA n-3 in hypolipidemic combination treatment: adding 3 to 3.6 g PUFA n-3 daily to pravastatin (40 mg/day) [21] or simvastatin (10 mg/day) [22] led to a decrease in LDL-C by 13–24% and TG by 27-30%. Double-blind study in patients with combined hyperlipidemia [23] showed, after the administration of EPA and DHA ethylesters in daily dose 1.68 g together with 10 mg atorvastatin, a significant increase in HDL-C (by 6% in comparison with atorvastatin alone). There was no further decrease in fasting TG and LDL-C after the addition of PUFA n-3. This treatment was connected with a decrease in the concentration of small dense LDL and postprandial hypertriglyceridemia (HTG) [23]. In another study, addition of PUFA n-3 concentrate (4 g/day) to simvastatin 10–40 mg daily for 24 weeks to patients with coronary heart disease (one third of them were diabetics) with persisting HTG led to a decrease in TG by 20–30% and VLDL-C by 30–40% [24]. At our clinic, we proved a favourable effect of the combination of PUFA n-3 with fibrate in the treatment of severe HTG [25].

In the present study, we used PUFA n-3 supplementation in patients with severe DDL that is resistant to long-term therapy even when using the combination of pravastatin 20 mg+micronised fenofibrate 200 mg/day. A significant and pronounced decrease in plasma TG was recorded. These changes were comparable with the results of Durrington et al. [24]. Increased lipoperoxidation, measured as a concentration of CD in LDL, which was found after 3 months of 3.6 g PUFA n-3 daily, indicates an elevation of minimally modified (oxidized) LDL in vivo.

Data from literature concerning oxidation stress influenced by PUFA n-3 are ambiguous. In the study of Dutch authors, PUFA n-3 raised the parameters of oxidation measured by the method of CD kinetics [26]. However, there are some studies in which indicators of oxidative stress influenced favourably [27], but diabetics were not usually included in these studies.

Addition of 3.6 g PUFA n-3 daily to the combined treatment with pravastatin and fenofibrate did not lead in our study to significant changes in fasting glycaemia and glycated haemoglobin concentrations. Effects of PUFA n-3 on glucose homeostasis have been summarized recently in a meta-analysis of Montori et al. [28], and it has been supposed that PUFA n-3 does not substantially aggravate glucose homeostasis.

The new and most important finding of this study was a decrease in plasma tHcy, connected with PUFA n-3 supplementation. In patients with DM type 2, higher levels of tHcy are associated with a higher prevalence of macroangiopathy, coronary heart disease and renal insufficiency [29].

Increased concentrations of tHcy are considered as a metabolic RF for many cardiovascular and noncardiovascular diseases [30]. Total Hcy seems to be a particularly strong predictor of cardiovascular events or death in subjects with preexisting illness such as coronary heart disease, DM or renal failure [31].

So far, there are not many data in the literature concerning the influence of PUFA n-3 on tHcy level. In 1993, Olszewski and McCully [11] published a study in which a 3-week administration of 12 g of fish oil daily led to a significant decrease in tHcy in 12 patients with hyperlipidemia type IIA and IIB. Grundt et al. [32] did not confirm this finding. The same authors have recently published a prospective study in which daily administration of 850 mg EPA+DHA to patients after acute myocardial infarction (12% of them were diabetics) led to a significant decrease in

tHcy without changes in the levels of CRP, ICAM-1 and E-selectin, as well as those of folate or cyanocobalamin; changes in tHcy concentrations did not correlate with changes in plasma FA composition [12]. In another study, carried out in a group of normolipidemics, an increase in tHcy was observed after administration of 6 g of PUFA n-3 daily [33]. The authors believed that PUFA n-3 could lead to a rise in NO concentration with a subsequent methionine synthase inhibition. Apparently, the relations between tHcy concentrations and PUFA n-3 are not clear and deserve a further detailed study.

It is known that fibrates as well as metformin increase tHcy concentrations [15,16]. Our patients were treated with both of these drugs. Therefore, favourable effects of PUFA n-3 consist not only in an additive hypolipidemic action but also, in this study setting, in tHcy lowering.

In our group of diabetics with severe DDL, we also noticed a significant decrease in MA after supplementation with PUFA n-3. This decrease significantly correlated with a rise in DHA and total PUFA n-3 in plasma TG.

Microalbuminuria in diabetics is an important RF for cardiovascular diseases, independent of other RFs such as hypertension or smoking [34]. In patients with DM type 2, higher excretion of albumin in urine is connected with ED and chronic inflammatory state; its progress in the course of time is significantly associated with risk of death [35].

Some authors consider MA to be a surrogate marker of generalized ED [36]. Hamazaki et al. [37] described a decrease in MA in DM type 2 after administration of EPA ethylester in a daily dose of 1.8 g for 6 months. These changes were associated with a significant increase in EPA in plasma. Perasallo et al. [38] found that diabetics with MA have a significantly lower content of PUFA n-6 in plasma TG.

The mechanism through which PUFA n-3 application leads to lowering of MA is not quite clear. Probably, it is connected with ED improvement, with a decrease in chronic inflammatory activity and TG. In patients with HTG, supplementation with PUFA n-3 resulted in a decrease in ICAM-1 and E-selectin [39]. In our patients, we did not notice any decreased concentrations of ICAM-1, TNF-α as well as IL-6 after PUFA n-3 administration (results not shown).

We suppose that changes in MA are not connected with those of oxidatively modified LDL, as, together with a decrease in MA, we observed an increased concentration of CD in LDL, which reflects concentrations of minimally modified LDL [18]. Serum concentrations of DHA and EPA correlated negatively with VCAM-1 and with the von Willebrand factor [40].

A further mechanism, connected with a decrease in MA, is the additive hypolipidemic effect of PUFA n-3. It is supposed that one of the reasons for ED is chronic HTG [41]. This assumption is supported by the findings of Kazumi et al. [42], who described decreased MA in HTG treated with fenofibrate.

PUFA n-3 is known to lower TG levels, but the effect of monounsaturated FA (oleic acid) on TG levels is inconsistent [43]. In our study, the level of TG, which was lowered after PUFA n-3 supplementation, tended to return to the baseline level. Supplementation with PUFA n-3 led to an insignificant increase in CD in LDL, which could be expected [44]. Substitution of PUFA n-3 for placebo (with oleic acid) caused a significant decrease in CD values. The antioxidative potential of oleic acid is widely accepted [45,46]. The reversal of tHcy values to the baseline after the placebo period strengthens the hypothesis about the causal relationship between PUFA n-3 supplementation and lowering of tHcy levels [12]. To the best of our knowledge, the changes in tHcy following oleic acid administration had not yet been studied.

In conclusion, the results of our pilot study show favourable effects of PUFA n-3 administration in severe DDL treated with a combination of statin–fibrate. The supplementation with PUFA n-3 to the combined statin–fibrate treatment in patients with severe DDL decreased TG and tHcy levels as well as MA. It could improve the cardiovascular risk profile and decrease the risk of atherothrombosis and result in a delay of diabetic nephropathy onset and progression. These findings should be proved in further studies with a larger group of patients.

Acknowledgments

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