Nutrition, Metabolism & Cardiovascular Diseases (2013) 23, 1066–1070

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/nmcd

Nutrition, Metabolism & Cardiovascular Diseases

Docosahexaenoic acid for the treatment of fatty liver: Randomised controlled trial in children☆

V. Nobili [a,*], A. Alisi [a], C. Della Corte [a], P. Risé [b], C. Galli [b], C. Agostoni [c], G. Bedogni [a,c,d]

[a] *Liver Research Unit, Bambino Gesù Children's Hospital and Research Institute, Roma, Italy*
[b] *Department of Pharmacological Sciences, University of Milano, Milano, Italy*
[c] *Department of Maternal and Pediatric Sciences, University of Milano, Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milano, Italy*
[d] *Clinical Epidemiology Unit, Liver Research Center, Basovizza, Trieste, Italy*

Received 29 May 2012; received in revised form 25 October 2012; accepted 30 October 2012
Available online 7 December 2012

KEYWORDS
Randomized controlled trial;
Long-chain polyunsaturated fatty acids;
Docosahexaenoic acid;
Children;
Adolescents;
Non-alcoholic fatty liver disease

Abstract *Background and aim:* Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in children. We tested whether dietary supplementation with docosahexaenoic acid (DHA) can decrease liver fat content in children with NAFLD.
Methods and results: We performed a randomized controlled trial of DHA supplementation (250 mg/day and 500 mg/day) *vs.* placebo in 60 children with NAFLD (20 children per group). The main outcome was the change in liver fat as detected by ultrasonography after 6, 12, 18 and 24 months of treatment. Secondary outcomes were changes in triglycerides, alanine transaminase (ALT), body mass index (BMI) and homeostasis model assessment of insulin resistance (HOMA). The odds of more severe versus less severe liver steatosis decreased to the same degree at 6 months in children treated with DHA 250 mg/day and DHA 500 mg/day *vs.* placebo and persisted virtually unmodified for 24 months (OR $\leq$ 0.02, $p \leq 0.05$ for all time points). Triglycerides were lower in the DHA groups than in the placebo group at any time point and ALT was lower in these groups from month 12 onwards. HOMA was lower in the DHA 250 mg group *vs.* placebo at months 6 and 12.
Conclusion: DHA supplementation improves liver steatosis in children with NAFLD. Doses of 250 mg/day and 500 mg/day of DHA appear to be equally effective in reducing liver fat content.

☆ **Trial identifier:** NCT00885313 (www.clinicaltrials.gov).

* Corresponding author. Liver Research Unit, Bambino Gesù Children's Hospital and Research Institute, Square S. Onofrio 4, 00165 Rome, Italy. Tel.: +39 06 68 59 22 43; fax: +39 06 68 59 21 92.
E-mail address: nobili66@yahoo.it (V. Nobili).

0939-4753/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.numecd.2012.10.010

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in children living in Western countries [1]. Although there is a general consensus that lifestyle changes should be the first step of NAFLD treatment in children as in adults [2—4], there is mounting interest in N-3 long-chain polyunsaturated fatty acids (LCPUFA) as potential treatment for liver steatosis [5,6].

Dietary N-3 LCPUFA lower blood triglycerides and have anti-inflammatory and insulin-sensitizing properties [5] which may explain why they are effective in reducing liver fat content [6]. We have recently reported the 6-month results of a randomized controlled trial (RCT) aimed at testing the efficacy of docosahexaenoic acid (DHA) on liver steatosis in children with NAFLD [7].

In the present paper, we report the long-term results of such RCT by showing the effect of DHA at 12, 18 and 24 months of follow-up.

Methods

Trial design

The present RCT, registered as NCT00885313 on www.clinicaltrials.gov, was performed on 60 consecutive children followed as outpatients at the Liver Research Unit of the Bambino Gesù Children Hospital (Rome, Italy) [7].

Children were eligible for the study if they had: 1) age $<$18 years, 2) persistently elevated serum alanine transaminase (ALT $\geq$ 40 U/L), 3) diffusely hyperechogenic liver at ultrasonography, and 4) liver biopsy consistent with NAFLD. Children were excluded from the study if they had any of the following: 1) viral liver disease, 2) autoimmune liver disease, 3) Wilson's disease, 4) α-1-antitrypsin deficiency, 5) celiac disease, 6) alcohol consumption (any quantity), 7) use of parenteral nutrition, 8) use of drugs known to induce fatty liver, and 9) previous use of N3-LCPUFA.

Twenty patients were treated with DHA 250 mg/day, 20 with DHA 500 mg/day and 20 with placebo. A balanced low-calorie diet was prescribed and physical activity was suggested to all patients as described in detail elsewhere [8]. Reinforcement of lifestyle changes was made by the first author at all visits.

Owing to the lack of data on the effects of DHA on liver steatosis in children, we choose doses of DHA known to be associated with physiologically relevant effects [7]. The presently suggested dietary intake of DHA for individuals at cardiovascular risk ranges between 250 and 500 mg/day [9], i.e. the two doses that we tested in this study. In detail, we hypothesized that the administration of placebo, DHA 250 mg/day and DHA 500 mg/day would increase whole blood DHA of a mean (standard deviation) of 0.0 (0.7)%, 0.5 (0.7)% and 1.0 (0.7)%, respectively. To detect this difference as significant at an alpha level of 0.05 with a power of 95%, 16 subjects per group were needed (analysis of variance with Monte Carlo simulation) [7]. To control for possible losses at follow-up, we enrolled 20 subjects per group.

A computer-generated randomization list assigned participants in equal number to DHA 250 mg/day, DHA 500 mg/day (39% DHA oil obtained from *Schyzochitrium*, Martek Biosciences Corporation, Columbia, MD, USA) and placebo (290 mg linoleic acid supplied with germ oil, Gelfipharma International, Lodi, Italy). A statistician, who did not perform the final analysis, generated the allocation sequence and assigned participants to the treatment groups. DHA and placebo pills were of the same number (1), size, appearance, taste and provided about 7 kcal of energy/pill (DMF, Lainate, Italy). Pills were stored at the hospital pharmacy and dispensed at the baseline visit and every 2 months thereafter. Participants, investigators and outcome assessors were blinded to the treatment for all the duration of the study.

The main outcome was the change in liver fat content as detected by ultrasonography after 6, 12, 18 and 24 months of treatment. Secondary outcomes were the changes in fasting triglycerides, ALT, HOMA and body mass index (BMI) after 6, 12, 18 and 24 months of treatment.

Compliance to the study treatment was evaluated by pill count at every visit, review of medication records, and direct interview of patients by the first author. Adverse events were defined as those injuries related to or caused by the study treatment. At each visit, parents were specifically asked about adverse events, and the first author checked for any association between treatment and adverse events.

The trial was approved by the Ethical Committee of the Bambino Gesù Children's Hospital and written informed consent was obtained from the parents or legal guardians of the children.

Liver ultrasonography

Liver ultrasonography was performed by an experienced radiologist using an Acuson Sequoia C512 scanner equipped with a 15L8 transducer (Universal Diagnostic Solutions, Oceanside, CA). Absent steatosis (grade 0) was defined as normal liver echo-texture; mild steatosis (grade 1) as slight and diffuse increase in fine parenchymal echoes with normal visualization of diaphragm and portal vein borders; moderate steatosis (grade 2) as moderate and diffuse increase in fine echoes with slightly impaired visualization of diaphragm and portal vein borders; and severe steatosis (grade 3) as fine echoes with poor or no visualization of diaphragm, portal vein borders and posterior portion of the right lobe [10,11].

Clinical and laboratory evaluation

Weight and height were measured following standard guidelines [12]. Body mass index (BMI) was calculated and converted to standard deviation scores (SDS) using the Centers for Disease Control 2000 reference data [13]. Glucose was measured by standard methods and insulin by means of radioimmunoassay (MYRIA Technogenetics, Milan, Italy). Blood fatty acids, including DHA, were analyzed in a drop of whole blood absorbed on a strip and transmethylated for gas-chromatography analysis [14].

Statistical analysis

Most variables were not normally distributed and all are reported as medians and interquartile ranges (IQR). IQR

was calculated as the difference between the 75th and 25th percentile. The effect of treatment on the main ordinal outcome (liver steatosis coded as 0, 1, 2 and 3) and the secondary continuous outcomes (triglycerides, ALT, BMI and HOMA) was evaluated using an ordinal generalized linear model (OGLM) and linear models employing treatment (0 = placebo, 1 = DHA 250 mg/day, 2 = DHA 500 mg/day; discrete), time (0, 6, 12, 18 and 24 months; discrete), a treatmentXtime interaction (discreteXdiscrete) and the baseline value of the outcome as predictors [15,16]. Repeated measures were taken into account by specifying cluster confidence intervals (CI) for each patient. The OGLM allows to directly control for heteroscedasticy and defaults to the proportional odds logistic regression model when the parallel-lines assumption is met [17,18]. The odds-ratio (OR) obtained from such model is a measure of the odds of more severe *vs.* less severe steatosis [19]. Between-group comparisons at each time point (6, 12, 18 and 24 months) were performed using pre-specified contrasts with Wald tests and Bonferroni's correction for multiple comparisons. Probabilities estimated from the OGLM and values estimated from the linear models were plotted to aid the clinical interpretation of the results. The analysis was intention to treat and there were no missing data. Statistical significance was set to a p-value <0.05 and all statistical tests were two-tailed. Statistical analysis was performed using Stata 12.1 (Stata Corp, College Station, TX, USA) together with the user-written oglm package [20].

Results

Table 1 reports the baseline measurements of the 60 children aged 6—16 years that were enrolled into the study.

As we reported in detail elsewhere [7], absolute increases of serum DHA equal to 0.65% (95%CI 0.30—1.01%) and 1.15% (95%CI 0.87—1.43%) were obtained at 6 months in the DHA 250 mg/day and 500 mg/day groups *vs.* placebo, respectively, matching the expectations we had when calculating sample size.

Fig. 1 plots the OR of more severe *vs.* less severe steatosis in the DHA groups *vs.* placebo. The OR was very small ($\leq$0.02) at any visit starting from month 6 ($p \leq 0.05$ for all values). Even if 95%CI were wider for the DHA 500 mg/day group, there is no doubt that there was a considerable reduction in the progression to more severe steatosis in the DHA groups as compared to placebo. On the contrary, no difference in such progression was noted between the DHA 250 mg/day and 500 mg/day groups ($p > 0.05$).

Fig. 2 plots the probability of liver steatosis as estimated from the OGLM whose OR were reported in Fig. 1. This is a cumulative probability, i.e. the probability of the four degrees of liver steatosis (0, 1, 2, and 3) sums to 1 at any time point. To interpret these data, one should start considering that all children had degree 2 or 3 liver steatosis at baseline (Table 1 and panels 2 and 3 of Fig. 2). As expected from Fig. 1, all changes were evident at 6 months and were stable thereafter. No patient in the placebo group had his/her fatty liver cleared while this happened for 10% and 15% of patients treated with DHA 500 and 250 mg respectively (Panel 0 of Fig. 1). Likewise, the number of patients "reverting" to stage 1 steatosis was no more than 20% in the placebo group but between 40% and 50% in the DHA groups (Panel 1 of Fig. 2). The probability of moderate steatosis increased up to 45% in the placebo group while it was at least 10% lower in the DHA groups (Panel 2 of Fig. 2). Lastly, the probability of degree 3 steatosis decreased only from 60% to 40% in the placebo group but from 60% to less than 10% in the DHA groups (panel 3 of Fig. 2).

Fig. 3 depicts the values of the secondary outcomes, i.e. triglycerides, ALT, BMI and HOMA. As for the main outcome, these changes were always comparable in the DHA 250 mg/day and DHA 500 mg/day groups ($p > 0.05$). Triglycerides

Table 1 Baseline measurements of the children. Values are median and interquartile range (IQR) for continuous variables and counts for categorical variables. IQR is calculated as the difference between the 75th and 25th percentiles. Abbreviations: DHA = docosahexaenoic acid; BMI = body mass index; SDS = standard deviation scores; ALT = alanine transaminase; HOMA = homeostasis model assessment of insulin resistance; US = ultrasonography.

	Placebo	DHA 250 mg/day	DHA 500 mg/day
N	20	20	20
Gender (M/F)	8/12	8/12	9/11
Age (years)	13 (4)	11 (3)	11 (2)
Weight (kg)	57 (24)	55 (15)	54 (15)
Height (m)	1.51 (0.28)	1.48 (0.20)	1.50 (0.18)
BMI (kg/m^2)	26.1 (5.1)	26.6 (4.9)	24.4 (3.6)
BMI (SDS)	1.76 (0.81)	1.81 (1.02)	1.63 (1.12)
DHA (%)	1.58 (0.41)	1.25 (0.44)	1.21 (0.32)
ALT (U/L)	78 (37)	70 (25)	57 (27)
Triglycerides (mg/dL)	89 (39)	90 (38)	89 (25)
Glucose (mg/dL)	87 (9)	86 (9)	81 (13)
Insulin (μU/mL)	11 (6)	12 (15)	10 (10)
HOMA	2.2 (1.3)	2.4 (3.2)	1.9 (1.5)
Liver Steatosis-US (0/1/2/3)	0/0/8/12	0/0/8/12	0/0/8/12

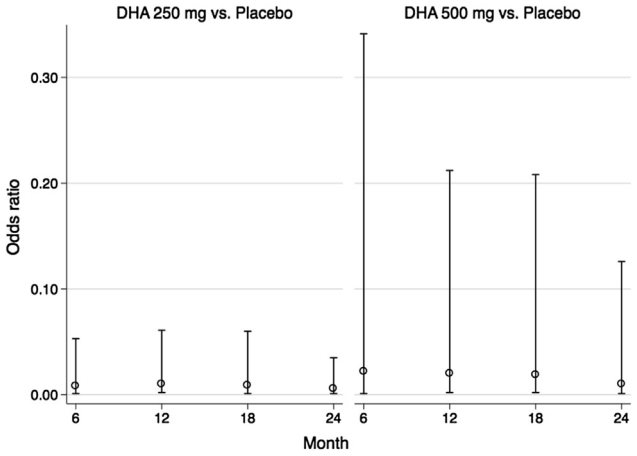

Figure 1 Odds ratio of more severe *vs.* less severe liver steatosis during the study. Odds ratios and 95% confidence intervals were obtained from an ordinal generalized model. Abbreviations: DHA = docosahexaenoic acid.

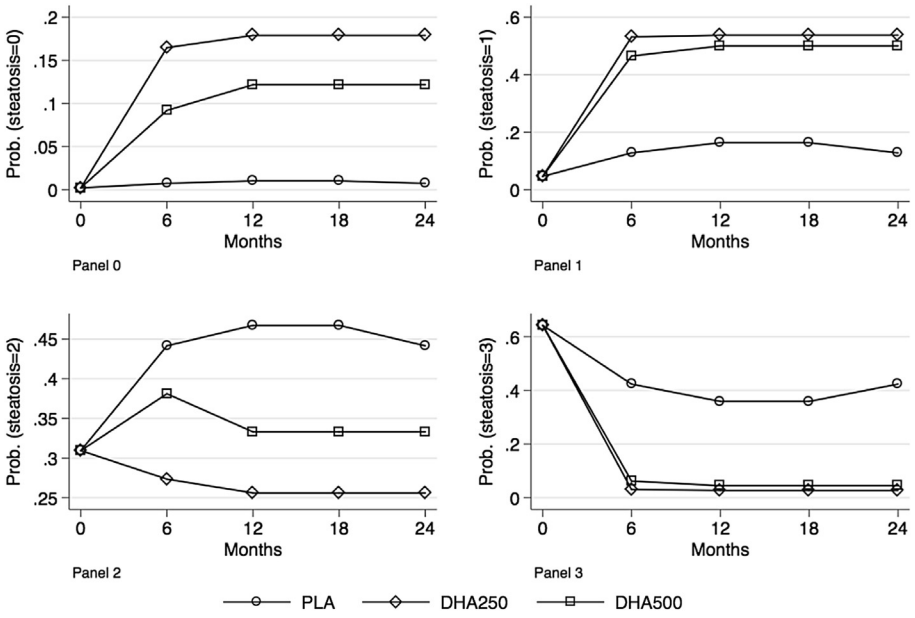

Figure 2 Probability of liver steatosis during the study. The depicted mean probabilities were obtained from the ordinal generalized linear model whose OR are plotted in Fig. 1.

were lower in the DHA groups than in the placebo group at any time point and ALT was lower in these groups from month 12 onwards ($p < 0.05$). BMI changes were always comparable in the placebo, DHA 250 mg and DHA 500 mg groups ($p > 0.05$). HOMA was lower in the DHA 250 mg group *vs.* placebo at months 6 and 12 ($p < 0.05$) but this was not found for the DHA 500 mg group.

Discussion

In this RCT, we found that a previously reported decrease in liver fat obtained at 6 months [7] persisted virtually unchanged after 24 months of supplementation with DHA. Moreover, DHA doses of 250 mg/day and 500 mg/day appeared to be equally effective in reducing liver steatosis.

Changes in triglycerides and HOMA went in the expected direction of a decrease in DHA-supplemented children but their estimates were imprecise and larger sample sizes are needed to adequately estimate these effects. We found no long-term effect of any dose of DHA on BMI and only a modest effect on ALT, which is compatible with available studies [6]. Also changes in weight and height, from which BMI is computed, were comparable between groups (data not shown). Contrarily to some trials [21] and in agreement with other trials [22], we found no effect of DHA on weight loss.

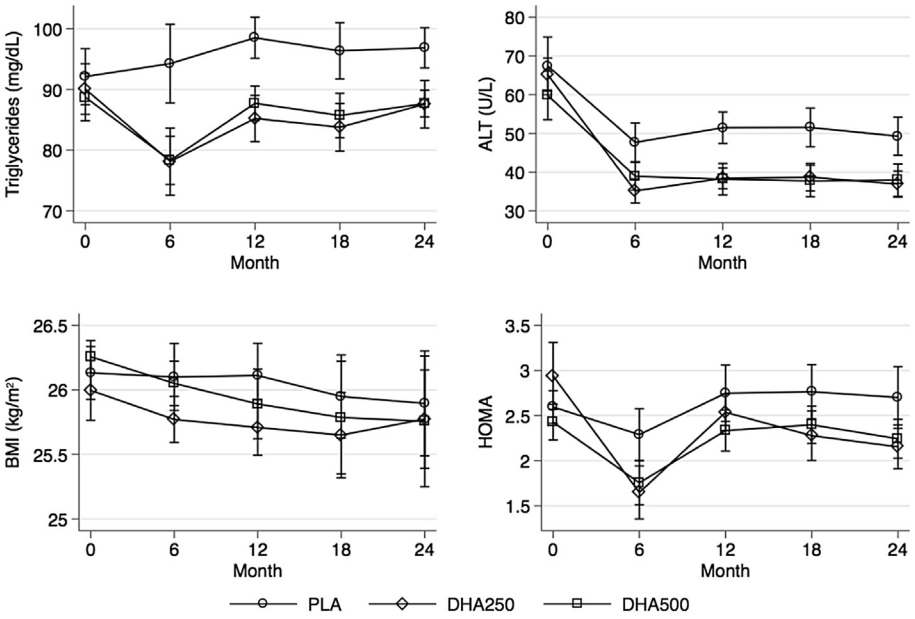

Figure 3 Changes in the secondary outcomes during the study. Abbreviations: ALT = alanine transaminase; BMI = body mass index; HOMA = homeostasis model assessment of insulin resistance.

However, we were interested in BMI not as main outcome but as potential confounder of the DHA-NAFLD relationship [6], something which is ruled out by our findings.

Although this is the first RCT evaluating the effects of DHA supplementation in pediatric NAFLD, it is not without limitations. While we performed liver biopsy at baseline to diagnose NAFLD [7], we used ultrasonography to evaluate the changes of liver steatosis. This was done because of the recent demonstration from our group that ultrasonography offers an accurate assessment of liver fat as compared to liver biopsy [11] and because of the ethical impossibility of performing repeated biopsies during the study. Also, while we performed an oral glucose tolerance test (OGTT) at the baseline and 6-month visits to evaluate insulin sensitivity through calculation of the insulin sensitivity index [7,10], we did not repeat the OGTT, again for ethical reasons, at further visits. Thus, although we found a decreasing trend for HOMA levels in the DHA 250 mg/day group, we could not test the long-term persistence of the clinically relevant decrease in ISI that we detected at 6 months [7].

Liver steatosis has been reported to regress frequently in the general population [23] and is especially sensitive to weight loss and physical activity [6]. Our RCT has many repeated measures, helping to evaluate the timing of the effect of DHA on liver steatosis. This effect appears to be at a peak at 6 months but it may be present sooner [6]. Further RCT should consider performing repeated measures between 0 and 6 months to allow a better characterization of the effect of DHA. Even if further studies in all ages of life are clearly needed to determine the optimal dose of N3-LCPUFA to reduce liver fat, our data agree with those available on adults in showing that higher doses are not necessarily more effective than lower doses [6]. Even more importantly, studies are needed to test the whether the reduction in liver fat achieved by DHA and other N3-LCPUFA is associated with hard clinical outcomes. Owing to the increasing evidence provided by cohort studies that fatty liver may be a predictor of type 2 diabetes mellitus and cardiovascular disease [3], intervention studies with N3-LCPUFA aimed at reducing liver fat content may be useful to test the existence of a cause—effect relationship between NAFLD and incident diabetes or cardiovascular disease.

In conclusion, DHA supplementation improves liver steatosis in children with NAFLD and doses of 250 mg/day and 500 mg/day appear equally effective.

References

[1] Alisi A, Nobili V. Non-alcoholic fatty liver disease in children now: lifestyle changes and pharmacologic treatments. Nutrition 2012;28:722—6.

[2] Keating SE, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol 2012;57:157—66.

[3] Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol 2010;53:372—84.

[4] Socha P, Horvath A, Vajro P, Dziechciarz P, Dhawan A, Szajewska H. Pharmacological interventions for nonalcoholic fatty liver disease in adults and in children: a systematic review. J Pediatr Gastroenterol Nutr 2009;48:587—96.

[5] Masterton GS, Plevris JN, Hayes PC. Review article: omega-3 fatty acids—a promising novel therapy for non-alcoholic fatty liver disease. Aliment Pharmacol Ther 2010;31:679—92.

[6] Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol 2012;56:944—51.

[7] Nobili V, Bedogni G, Alisi A, Pietrobattista A, Risé P, Galli C, et al. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. Arch Dis Child 2011;96:350—3.

[8] Nobili V, Marcellini M, Devito R, Ciampalini P, Piemonte F, Comparcola D, et al. NAFLD in children: a prospective clinical-pathological study and effect of lifestyle advice. Hepatology 2006;44:458—65.

[9] EFSA Panel on Dietetic Products. Nutrition and allergies (NDA). Scientific opinion on the tolerable upper intake level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). EFSA J 2012;10:2815—48.

[10] Bedogni G, Gastaldelli A, Manco M, De Col A, Agosti F, Tiribelli C, et al. Relationship between fatty liver and glucose metabolism: a cross-sectional study in 571 obese children. Nutr Metab Cardiovasc Dis 2012;22:120—6.

[11] Shannon A, Alkhouri N, Carter-Kent C, Monti L, Devito R, Lopez R, et al. Ultrasonographic quantitative estimation of hepatic steatosis in children with NAFLD. J Pediatr Gastroenterol Nutr 2011;53:190—5.

[12] Lohman TG, Roche AF, Martorell R. Anthropometric standardization reference manual. Champaign, IL: Human Kinetics Books; 1988.

[13] Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, et al. CDC growth charts: United States. Adv Data 2000:1—27.

[14] Marangoni F, Colombo C, Galli C. A method for the direct evaluation of the fatty acid status in a drop of blood from a fingertip in humans: applicability to nutritional and epidemiological studies. Anal Biochem 2004;326:267—72.

[15] Rabe-Hesketh S, Skrondal A. Multilevel and longitudinal modeling using Stata. continuous responses, vol. 1. College Station, TX: Stata Press; 2012.

[16] Rabe-Hesketh S, Skrondal A. Multilevel and longitudinal modeling using Stata. categorical responses, counts and survival, vol. 2. College Station, TX: Stata Press; 2012.

[17] Williams R. Fitting heterogeneous choice models with OGLM. Stata J 2010;10:540—67.

[18] Agresti A. Analysis of ordinal categorical data. Hoboken, N.J: Wiley; 2010.

[19] Bedogni G, Kahn HS, Bellentani S, Tiribelli C. A simple index of lipid overaccumulation is a good marker of liver steatosis. BMC Gastroenterol 2010;10:98.

[20] Williams R. Generalized ordered logit/partial proportional odds models for ordinal dependent variables. Stata J 2006;6:58—82.

[21] Thorsdottir I, Tomasson H, Gunnarsdottir I, Gisladottir E, Kiely M, Parra MD, et al. Randomized trial of weight-loss-diets for young adults varying in fish and fish oil content. Int J Obes Relat Metab Disord 2007;31:1560—6.

[22] DeFina LF, Marcoux LG, Devers SM, Cleaver JP, Willis BL. Effects of omega-3 supplementation in combination with diet and exercise on weight loss and body composition. Am J Clin Nutr 2011;93:455—62.

[23] Bedogni G, Miglioli L, Masutti F, Castiglione A, Crocè LS, Tiribelli C, et al. Incidence and natural course of fatty liver in the general population: the Dionysos study. Hepatology 2007;46:1387—91.