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Efficacy of docosahexaenoic acid-choline-vitamin E (DHA-CHO-VE) in paediatric NASH: a randomized controlled clinical trial

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Running title: Combination therapy in paediatric NASH

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Abstract

Non-alcoholic steatohepatitis (NASH), a progressive form of non-alcoholic fatty liver disease (NAFLD), is one of the most common hepatic diseases in children. We conducted a randomized controlled clinical trial on children with biopsy-proven NASH based on a combinatorial nutritional approach compared to placebo.

Participants were assigned to lifestyle modification plus placebo, or lifestyle modification plus a mix containing docosahexaenoic acid, choline and vitamin E (DHA-CHO-VE). Forty children and adolescents concluded the trial. The primary outcome was the improvement of liver hyperechogenicity. Secondary outcomes included alterations of ALT and other metabolic parameters. Furthermore, changes of serum bile acids (BA) and plasma fibroblast growth factor 19 (FGF19) levels were evaluated as inverse biomarkers of disease severity.

At the end of the study, we observed a significant decrease in severe steatosis in the treatment group (50% to 5%, p=0.001). Furthermore, although the anthropometric and biochemical measurements in the placebo and DHA-CHO-VE groups were comparable at baseline, at the end of the study ALT and fasting glucose levels improved only in the treatment group. Finally, we found that BA levels were not influenced whereas FGF19 levels were significantly increased by DHA-CHO-VE.

The results suggest that a combination of DHA, vitamin E and choline could improve steatosis and reduce ALT and glucose levels in children with NASH. However, further studies are needed to assess the impact of a DHA and vitamin E combination on repair of liver damage in paediatric NASH.

Key words: bile acids, choline; docosahexaenoic acid; FGF19; non-alcoholic fatty liver disease; steatosis; vitamin E.
Introduction

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial disease that is becoming more prevalent due to a rapid rise in obesity. In fact, NAFLD is currently the most common chronic hepatopathy in both adults and children in most of the western world (Loomba and Sanyal 2013; Nobili et al. 2013). Besides obesity, other factors affect the prevalence of paediatric NAFLD, including genetic susceptibility, social-environmental variables and dysmetabolism (Bedogni et al. 2005). Metabolic syndrome traits, including insulin resistance (IR), dyslipidaemia and type two diabetes, are well known risk factors associated with NAFLD in children (Nobili et al. 2015; Nobili et al. 2016).

Since in children with NAFLD clear guidelines on pharmacological interventions are still lacking, lifestyle interventions designed to reduce body weight, including diet and exercise, remain the first-line treatments (Africa et al. 2016). However, several lines of evidence demonstrate that in children, as well as in adults, lifestyle interventions improve only metabolic parameters and simple steatosis but not the histological features of non-alcoholic steatohepatitis (NASH), which is the most severe form of NAFLD (Vilar-Gomez et al. 2015; Nobili et al. 2008). Therefore, during the last several decade drugs acting upon mechanisms that are involved in the pathogenesis of NAFLD, including insulin resistance (IR) and oxidative stress, also have been tested in paediatric settings (Nobili et al. 2008; Lavine et al. 2011).

To discover the medication and dose that are the best for children with NASH, while avoiding adverse pharmacological effects, recent studies have evaluated the effects of nutritional supplementation, such as vitamins, probiotics and omega-3 fatty acids (Nobili et al. 2013; Nobili et al. 2014a; Alisi et al. 2014; Janczyk et al. 2015). Lavine et al. (2011) have reported that vitamin E favourably affects hepatocellular ballooning. We have demonstrated that treatment with docosahexaenoic acid (DHA), an omega-3 fatty acid, may improve liver steatosis, insulin sensitivity and hepatocellular necro-inflammation in children with NASH (Nobili et al. 2013; Nobili et al. 2014a). However, none of the tested therapeutic approaches has proved entirely satisfactory,
suggesting that a mix of multiple drugs and/or dietary supplements targeting specific pathogenic features could be more effective (Alisi et al. 2012).

The liver is centrally responsible for the metabolism of choline, an essential nutrient. Choline is mainly phosphorylated and used to make phospholipids, or oxidized and used as a major source of methyl groups (Corbin and Zeisel 2012). An important choline metabolite in liver is phosphatidylcholine (PC), required for the generation of very low density lipoprotein (VLDL), the lipoprotein involved in the export of fat from liver. Impaired biosynthesis of VLDL is a crucial mechanism in hepatic steatosis development (Vance 2008). In addition, PC is a major component of cellular membranes and decrease in PC synthesis can impair the integrity of the hepatocyte membrane, leading to ALT increase and NASH (Li et al. 2006). However, choline is derived not only from the diet. In fact, endogenous biosynthesis of PC may occur in liver, catalysed by phosphatidylethanolamine N-methyltransferase (PEMT) (Corbin and Zeisel 2012). Moreover, choline may be also oxidized to betaine in humans; both betaine and choline are methyl donors in pathways involving the re-methylation of homocysteine to methionine, thereby reducing blood levels of homocysteine, which are positively correlated with NAFLD severity in both adults and children (de Carvalho et al. 2013; Pastore et al. 2014). These findings, together with experimental studies demonstrating that a choline-deficient diet may predispose to NAFLD in rodent models, strongly suggest that choline supplementation could ameliorate liver damage in NAFLD (Al Rajabi et al. 2014).

Here, we report the results of a randomized clinical trial to investigate the effect on paediatric NAFLD of these mixed compounds compared with placebo.
Patients and Methods

Study population and design

Sixty Italian children or adolescents (age range, 4-16 years) referred to the Hepato-metabolic Disease Unit of Bambino Gesù Children Hospital (Rome, Italy) with liver biopsy-proven NASH and without other causes of liver disease were screened for the study. Excluded were patients with Wilson disease, hepatitis B and C, acute systemic disease, autoimmune hepatitis, hypothyroidism, cystic fibrosis, celiac disease, suspected muscular dystrophy, alpha-1-antitrypsin deficiency and other metabolic inherited diseases. Patients were also excluded if body weight and carbohydrate metabolism were altered by parenteral nutrition, protein malnutrition, previous gastrointestinal surgery, structural abnormalities of the gastrointestinal tract or neurological impairment. Finally, the use of nonsteroidal anti-inflammatory drugs, antibiotics, probiotics or antisecretory drugs capable of causing achlorhydria within the two months before enrolment was also an exclusion criterion. Forty-three of 60 screened children with NAFLD met these inclusion criteria: age 4-16 years [13 (30.2%) children and 30 (69.8%) adolescents], persistently elevated serum aminotransferase levels, diffusely echogenic liver on imaging studies suggestive of fatty liver and biopsy findings consistent with NASH. All these children were enrolled in this placebo-controlled clinical trial.

Children were randomized by computer to receive every day for six months either pearls combining 250 mg of DHA, 39 UI of vitamin E and 201 mg of choline (DHA-CHO-VE; Pro DHA Steatolip Plus®) or externally identical pearls as placebo. Both were provided by DMF Dietetic Metabolic Food (Italy). Concomitantly, all patients were recommended to follow a hypocaloric diet (25-30 kcal/kg/day) and to engage in twice weekly 1-hour physical activity during the treatment and for further 6 months of follow-up. Compliance with treatment and diet was monitored on monthly visits by counting the amount of investigational product left in the bottle.

Complete medical histories were recorded for all participants. Anthropometrical data, biochemical data and liver ultrasound findings were collected at baseline and after 12 months from trial start. Patients and investigators were blinded before and after intervention assignment.
Symptoms and side effects were assessed at each visit by an investigator. Adverse events were defined as injuries caused by the treatments under study. At each visit, parents were specifically asked about adverse events, and the first author checked for any association between the adverse events and morbidity.

Clinical and research investigators of the Bambino Gesù Children Hospital designed the study and managed the DHA-CHO-VE trial. The Hospital Ethics Research Committee approved the study, in accordance with the Declaration of Helsinki (as revised in Seoul, Korea, October 2008), and monitored that all experimental procedures were carried out in accordance with the relevant guidelines included in the protocol. Parents of the included patients gave their written informed consent to therapies, to the tests performed and to publication of the results. The study was registered at ClinicalTrials.gov as NCT01934777 on 30 August 2013. However, we underline that the current primary endpoint differs from that in the protocol submitted to ClinicalTrials.gov because liver biopsy at 12 months in the placebo group was not performed for ethical reasons. The original trial, in fact, had as primary endpoint improvement in NAFLD Activity Score (NAS) and as secondary endpoints all parameters of metabolic syndrome and bright liver at ultrasonography. Physicians responsible for patients decided to change the primary endpoint after the trial had begun but when data were still blinded. Thus, at the end of the trial (12 months), each participant/guardian was informed of his or her group in the study and, if he or she belonged to the DHA-CHO-VE group, we proposed a second liver biopsy. Consequently, we compared, as primary endpoint, improvement in liver hyperechogenicity in the DHA-CHO-VE group with that in the placebo group after 12 months. The secondary endpoints were amelioration in ALT and in other metabolic parameters in both groups. The safety of the mixture was also evaluated, monitoring possible side effects.

*Anthropometrics*
Anthropometric data were collected at baseline and after 12 months. Weight was measured using a conventional scale with a precision of 100 g and height was measured by a Harpenden stadiometer with a precision of 1 mm. Body mass index (BMI) was expressed in kilograms per square meters (kg/m²). Waist circumference (WC) was evaluated by using a tape to the nearest 0.5 cm measure, at the narrowest circumference between the lower costal margin and the iliac crest in standing position.

Laboratory parameters

Blood collected after overnight fasting at baseline and at month 12 was immediately analysed by standard laboratory methods for alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl-transferase (GGT) activity and for total triglycerides, cholesterol, glucose and insulin. Insulin resistance was evaluated by the homeostatic model assessment (HOMA) equation [fasting insulin (µU/mL) X fasting glucose (mg/dL)/405] (Matthews et al. 1985).

Ultrasound

The same blinded physician evaluated ultrasound (US) liver brightness at baseline and after the 12-month treatment. Liver US was performed by an experienced radiologist, using an Acuson Sequoia C512 scanner equipped with a 15L8 transducer (Universal Diagnostic Solutions, Oceanside, CA). Normal liver without steatosis (grade 0) was defined as having normal liver echotexture; mild steatosis (grade 1) as slight and diffuse increase in fine parenchymal echoes with normal visualisation of diaphragm and portal vein borders; moderate steatosis (grade 2) as a moderate and diffuse increase in fine echoes with slightly impaired visualisation of diaphragm and portal vein borders; and severe steatosis (grade 3) as fine echoes with poor or no visualisation of diaphragm, portal vein borders and posterior portion of the right lobe.
Liver biopsy

As already stated, for ethical reasons set out in the position paper of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (Vajro et al. 2012), liver biopsy was performed at baseline (T0) in both groups and after 12 months only in the treatment arm.

US-guided liver biopsies were fixed in 10% buffered formalin and interpreted by an experienced pathologist (RDV) employing criteria proposed by the NAFLD Clinical Research Network (Kleiner et al. 2005). Steatosis was graded 0–3 (0 = <5% steatosis, 1 = 5–33%, 2 = 33–66% and grade 3 = >66%); lobular inflammation was scored by number of inflammatory foci per 200 per field (0 = no inflammatory foci, 1 = <2 foci; 2 = 2–4 foci and 3 = >4 foci); ballooning was graded 0–2 (0 = none; 1 = few balloon cells present and 2 = prominent ballooning). Portal inflammation (PI; 0 = no PI, 1 = mild PI, 2 = more than mild) was scored as proposed by Brunt et al. (2009). Fibrosis was staged 0–4 (0 = no fibrosis; 1 = periportal or perisinusoidal; 1A = mild, Zone 3, perisinusoidal; 1B = moderate, Zone 3, perisinusoidal; 1C = portal/periportal; 2 = perisinusoidal and portal/periportal; 3 = bridging fibrosis and 4 = cirrhosis). A NAS > 5 was used for further comparisons with variables of interest.

Intestinal fibroblast growth factor 19 assay

Blood was centrifuged at 2000 g for 12 min and plasma was stored at -80°C pending further analysis. A specific sandwich enzyme-linked immunosorbent assay was used to determine fibroblast growth factor 19 (FGF19) levels (Biovendor; Brno; Czech Republic).

Bile acids quantification

For bile acids (BA) quantitation, blood samples were collected from patients after 12 hours of fasting, with sera immediately separated and stored at -80 °C until assays. Total BA (tBA) and individual BA levels, including unconjugated and taurine (T)- and glycine (G)- conjugated species, were measured using high-performance liquid chromatography coupled with tandem mass...
spectrometry (HPLC-MS/MS). A full 15-BA profile was determined using 10 µl of serum; it included cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic (DCA), lithocholic (LCA), and ursodeoxycholic (UDCA) acid and their T-conjugates TCDCA, TLCA, TDCA, TCA and TUDCA and G-conjugates GCA, GCDCA, GLCA, GDCA and GUDCA. Individual BA were separated by HPLC using a reversed-phase C18 column with a methanol and water gradient. Deuterium-labeled internal standards served for quantification. Mass spectrometer Q Exactive™ MS/MS (Thermo Fisher Scientific, Waltham, MA) and high-performance quadrupole precursor selection with high-resolution and accurate-mass (HR/AM) Orbitrap™ detection were used for identification of single BA.

Statistical analysis

The SPSS Statistics package 21 (IBM SPSS, Armonk, NY) was used for statistical analyses of data. Patient characteristics and biochemical variables are expressed by mean ± standard deviation (SD) or number (%) as appropriate. The p value used for checking significance level was <0.05. To compare all studied variables between groups, independent sample t-testing for quantitative data was used. Comparisons between groups were performed using analysis of variance. Difference between proportions were evaluated by Chi-square testing. Pearson's correlation test was used to evaluate a possible correlation between BA and FGF19 or biochemical variables and histological pattern in NAFLD patients. Furthermore, multivariate regression analysis was used to test the independence of associations between steatosis and anthropometrics and laboratory parameters.
Results

Effect of DHA-CHO-VE on liver histology

Forty children with biopsy-proven NASH (of 60 screened and 43 enrolled) completed the trial (Supplementary Figure S1). No adverse events were reported. Table 1 shows all histological traits and score assessed at baseline. Histological traits did not differ significantly between placebo and treatment group at baseline. Comparing the treatment group over the time, we found some interesting differences. In particular, we found a significant improvement in steatosis (p = 0.01), ballooning (p = 0.001) and NAS (p = 0.0003) at 12 months compared to baseline data (Table 2). Second liver biopsy was not offered to the placebo group.

Effect of treatments on liver echogenicity and metabolic parameters

Changes in hepatic US findings differed between the placebo and DHA-CHO-VE groups. Patients in the DHA-CHO-VE group exhibited significant improvement in liver steatosis at 12 months (Table 3), with severe steatosis significantly decreased from 50% to 5% of patients (p=0.001).

Anthropometric and biochemical parameters were similar in the placebo and DHA-CHO-VE groups at entry (Table 4). However, after 12 months, statistically significant improvement in ALT and fasting glucose levels was found in the treatment group only.

Effect of treatment on BA metabolism

Serum BA levels are low in non-fibrotic and fibrotic patients with NAFLD potentially caused by metabolic changes, particularly by hyperinsulinemia (Jahnel et al. 2015). Al Rajabi et al. (2014) demonstrated that choline supplementation normalized hepatic cholesterol levels and circulating levels of BA, thus preventing progression to NASH and liver failure in Pemt+/−/Ldlr+/− mice on a high fat diet. Therefore, we evaluated if DHA-CHO-VE supplementation restored serum
levels of BA. As shown in Figure 1, mean levels of serum BA after 12 months of DHA-CHO-VE treatment were similar to those observed at baseline both in placebo (1.48±1.21 µM ; 1.91±1.85 µM) and in treatment group (1.97±1.91 µM ; 1.10±1.07 µM). No significant changes after 12 months were seen in G- and T-conjugates in the placebo group (G-conjugates: 1.02±1.01 µM; 0.80±0.79 µM and T-conjugates: 0.03±0.02 µM; 0.05±0.02 µM) or after DHA-CHO-VE treatment (G-conjugates: 1.23±1.11 µM; 0.55±0.47µM and T-conjugates: 0.08±0.04 µM; 0.01±0.01 µM).

Effect on FGF19 circulating levels

Circulating FGF19 levels are inversely correlated with fibrosis grade and positively correlated with UDCA levels in children with NAFLD (Alisi et al. 2013; Jahnel et al. 2015). Moreover, FGF19 may lower serum glucose levels in diabetic mice (Fu et al. 2004). Therefore, we analysed FGF19 circulating levels and investigated their possible correlations with fasting insulin, fasting glucose and individual BA in placebo and DHA-CHO-VE groups. In the placebo group, we found that FGF19 levels were similar at baseline and after 12 months: 41.3±18.9 pg/mL (p =0.14) ; 56.6±33.9 pg/mL (Figure 2). By contrast, in the DHA-CHO-VE group FGF19 levels were significantly (p <0.0001) higher after 12 months (110.1±45.3 pg/mL) than at baseline (46.8±28.1 pg/mL) (Figure 2). No statistically significant correlation was found between serum total BA levels and improvement in liver steatosis or in metabolic parameters. However, Pearson's correlation analysis with individual BA showed that UDCA levels were positively associated with FGF19 levels (0.828; p <0.0001).
Discussion

In this study, we evaluated the efficacy and safety of DHA-CHO-VE in the treatment of obese children with NASH. The results showed that, at the end of 6 months of treatment and 6 months of follow-up, the DHA, choline and vitamin E mixture improved steatosis, ALT activity and fasting glucose and was not associated with any adverse events. The effect on the histologic pattern of NASH was evaluated only in patients undergoing DHA-CHO-VE treatment, who at 12 months showed a significant improvement in steatosis, ballooning and NAS.

To the best of our knowledge, this is the first study that investigates the effects of a mix of three different nutritional supplements combined with diet and exercise on paediatric NAFLD. Previous clinical studies, in fact, were based on a single treatment combined with lifestyle intervention (Nobili et al. 2008; Lavine et al. 2011; Nobili et al. 2013). We previously reported that vitamin E alone improved liver function and promoted normalisation of ALT levels but it had no effect on hepatic histopathologic features of injury associated with paediatric NAFLD (Nobili et al. 2008; Nobili et al. 2006). By contrast, treatment of NAFLD In Children study reported that vitamin E administration, though not reducing ALT levels, improved hepatocellular ballooning in children with NASH (Lavine et al. 2011). We have demonstrated that the omega-3 DHA improved liver steatosis, ALT levels, triglyceride levels and IR in paediatric NAFLD in the absence of significant changes in BMI (Nobili et al. 2013). Furthermore, treatment of NASH children with 250 mg/day DHA after 18 months ameliorated hepatocyte steatosis, ballooning and NAS. Based on these findings we hypothesised that the combination between vitamin E and DHA could be more effective than a single therapy. Furthermore, we decided to add choline since its supplementation may increase PC-DHA plasma levels (da Costa et al. 2011) and hepatic concentrations of vitamin E (Borges et al. 2015).

Our results showed that DHA-CHO-VE combination therapy was able to induce a partial recovery of both steatosis and ballooning, a reduction in NAS and amelioration of ALT and fasting glucose levels. These results are similar to those previously observed with DHA alone (Nobili et al.
2013). However, without a parallel single-agent treatment group we cannot exclude that a dose of vitamin E lower than in previous studies (39IU vs. 600-800IU), along with great variation in dietary intake of choline, may partially have hidden the effect of supplemental vitamin E and choline. An additional hypothesis for the apparent lack of an additive effect of choline could be the presence of single nucleotide polymorphisms in \textit{PEMT} that impair enzyme activity (da Costa et al. 2011), as it occurs in subjects with patatin-like phospholipase domain-containing protein 3 gene variants who are not responders to DHA (Nobili et al. 2014b). In \textit{Pemt}^{-/-}/\textit{Ldlr}^{-/-} mice, choline supplementation restored normal levels of circulating levels of BA and prevented NASH progression (Al Rajabi et al. 2014). We found that circulating BA levels in both the placebo and the DHA-CHO-VE groups, at baseline as well as at 12 months, were lower than levels in healthy children (Jahnel et al. 2015). However, BA levels remain unchanged in both placebo and DHA-CHO-VE groups.

BAs are complex regulatory molecules (Fiorucci et al. 2009; Chiang 2013). In the liver, both free and conjugated BA bind to the ligand-binding domain of farnesoid X receptor (FXR), inhibiting transcription of \textit{CYP7A1}, which encodes the rate-limiting enzyme in BA synthesis (Chiang 2009; de Aguiar Vallim et al. 2013). In the endocrine pathway, intestinal BA activate FXR, inducing FGF19 synthesis/release, which, in turn, may be transported to the liver to activate the FGFR4/Klotho-β receptor, inhibiting BA \textit{de novo} synthesis (Holt et al. 2003; Wu et al. 2007; Lin et al. 2007). Studies in humans and mice revealed a link between impaired glucose metabolism and disruption of BA signalling (Lefebvre et al. 2009). Additionally, FXR deficiency is associated with IR and impaired glucose tolerance in mice (Cariou et al. 2006; Zhang et al. 2006). Human and animal studies found an association between impaired glucose metabolism and disruption of BA signalling in diabetes (Duran-Sandoval et al. 2004). Activation of FXR in insulin-resistant \textit{ob/ob} mice by the FXR agonist GW4064 led to reduced hyperinsulinemia and improved glucose tolerance (Cariou et al. 2006). Moreover, diabetic \textit{fa/fa} rats receiving the FXR agonist 6-ethyl-CDCA showed an improvement in insulin resistance in liver and skeletal muscle (Fiorucci et al. 2011). In patients treated with DHA-CHO-VE, we found an improvement in fasting glucose without any association
between BA levels and fasting glucose or insulin. However, we found that UDCA positively correlated with circulating levels of FGF19. Furthermore, our results demonstrated that FGF19 levels were significantly higher in the DHA-CHO-VE group after 12 months. These data accord with our previous studies reporting diminished FGF19 levels in paediatric NAFLD and FGF19 levels inversely correlated with progression to liver fibrosis but positively correlated with UDCA levels (Alisi et al. 2013; Jahnel et al. 2015). By contrast, high FGF19 levels in the treatment group may suppress BA de novo synthesis via different pathways (Wu et al. 2011), and short term UDCA administration may stimulate BA synthesis by reducing circulating FGF19 and FXR activation (Mueller et al. 2015). Therefore, the recurrent positive association between UDCA and FGF19 levels that we found in children with NAFLD is still far from being explained.

This study displays some limitations: i) the lack of an end-of-study liver biopsy in the placebo group (avoided for ethical reasons); ii) inability to separate the individual effects of DHA, vitamin E and choline; iii) inability to perform sample size calculation. However, it is the first clinical trial to combine choline with two agents known to be efficacious (DHA and vitamin E) in a paediatric setting (Lavine et al. 2011; Nobili et al. 2013) and its results demonstrate that DHA-CHO-VE combination therapy is well-tolerated and is able to improve steatosis, ALT and fasting glucose levels in children with NASH. Further long-term studies, as well as studies addressing genetic contributions to individual variation in drug response, are needed to assess the impact of a DHA, vitamin E and choline combination on repair of liver damage and hepato-metabolic alterations in paediatric NASH.

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Conflict of Interest: The authors have no conflict of interest to disclose.

Contributions
V.N. designed the study; E.Z., A.A., J.J. and A.C. performed analyses; A.M. and C.D.C. collected and analysed patient data; EZ, A.A., G.F. and V.N. wrote and reviewed the manuscript. All authors approved the final version of the article, including the authorship list.

Acknowledgement
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Table 1: Histology in the treatment group vs. placebo group at baseline.

<table>
<thead>
<tr>
<th>Histological parameters</th>
<th>PLACEBO</th>
<th>DHA-CHO-VE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td><strong>Steatosis</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
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<td>7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td><strong>Lobular Inflammation</strong></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><strong>Portal Inflammation</strong></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td><strong>Ballooning</strong></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td><strong>Fibrosis</strong></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<tr>
<td><strong>NAFLD Activity Score (NAS)</strong></td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
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Table 2. Improvement in histologic features, DHA-CHO-VE group.

<table>
<thead>
<tr>
<th>Histological parameters</th>
<th>Baseline</th>
<th>12 months</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>1.85 (0.72)</td>
<td>1.05 (0.97)</td>
<td>0.01</td>
</tr>
<tr>
<td>Lobular Inflammation</td>
<td>1.15 (0.48)</td>
<td>1.00 (0.32)</td>
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<td>Portal Inflammation</td>
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<td>Ballooning</td>
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<td>Fibrosis</td>
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<tr>
<td>NAS</td>
<td>4.35 (1.10)</td>
<td>2.65 (1.49)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Data are shown with mean ± standard deviation (SD). p<0.05 was considered significant. NAS = NAFLD Activity Score.
Table 3. Liver steatosis by ultrasound at baseline and after 12 months in both treatment groups.

<table>
<thead>
<tr>
<th>%</th>
<th>PLACEBO</th>
<th>DHA-CHO-VE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 months</td>
</tr>
<tr>
<td>No steatosis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td>Moderate</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Severe</td>
<td>35</td>
<td>15</td>
</tr>
</tbody>
</table>

*Chi-square test, p<0.05 was considered significant.
Table 4. Clinical and laboratory variables in placebo and treatment groups.

<table>
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<tr>
<th></th>
<th>PLACEBO</th>
<th>TREATMENT</th>
<th>p</th>
<th>Baseline</th>
<th>12 months</th>
<th>p</th>
<th>Baseline</th>
<th>12 months</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>10/10</td>
<td>10/10</td>
<td>-</td>
<td>14/6</td>
<td>14/6</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>13.2 (2.1)</td>
<td>14.2 (2.1)</td>
<td>0.15</td>
<td>13.2 (2.3)</td>
<td>14.2 (2.3)</td>
<td>0.19</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>66.3 (15.1)</td>
<td>66.4 (15.3)</td>
<td>0.97</td>
<td>68.0 (18.7)</td>
<td>67.3 (14.8)</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>28.3 (5.3)</td>
<td>28.1 (5.2)</td>
<td>0.90</td>
<td>27.6 (4.0)</td>
<td>27.7 (4.9)</td>
<td>0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WC, cm</strong></td>
<td>89.9 (9.7)</td>
<td>89.1 (9.5)</td>
<td>0.79</td>
<td>86.7 (10.3)</td>
<td>90.9 (10.0)</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AST, UI/L</strong></td>
<td>33.1 (18.3)</td>
<td>34.6 (36.3)</td>
<td>0.87</td>
<td>36.2 (13.0)</td>
<td>31.2 (15.0)</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ALT (UI/L)</strong></td>
<td>51.2 (51.6)</td>
<td>32.5 (17.8)</td>
<td>0.14</td>
<td>35.3 (32.6)</td>
<td>35.3 (20.7)</td>
<td>0.04</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Uric acid (mg/dL)</strong></td>
<td>6.5 (3.0)</td>
<td>10.5 (17.4)</td>
<td>0.33</td>
<td>5.3 (0.9)</td>
<td>5.2 (1.0)</td>
<td>0.80</td>
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</tr>
<tr>
<td><strong>Total cholesterol (mg/dL)</strong></td>
<td>154.5 (30.1)</td>
<td>143.4 (17.9)</td>
<td>0.17</td>
<td>150.1 (31.5)</td>
<td>146.5 (28.4)</td>
<td>0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDL cholesterol (mg/dL)</strong></td>
<td>100.8 (37.6)</td>
<td>94.5 (29.7)</td>
<td>0.57</td>
<td>84.0 (29.4)</td>
<td>76.1 (23.7)</td>
<td>0.37</td>
<td></td>
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<tr>
<td><strong>HDL cholesterol (mg/dL)</strong></td>
<td>46.6 (8.3)</td>
<td>48.6 (8.2)</td>
<td>0.46</td>
<td>47.3 (7.9)</td>
<td>49.65 (11.9)</td>
<td>0.48</td>
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<td></td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
<td>87.2 (46.2)</td>
<td>81.5 (37.2)</td>
<td>0.68</td>
<td>101.2 (51.3)</td>
<td>82.1 (47.6)</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose (mg/dL)</strong></td>
<td>82.5 (7.2)</td>
<td>80.1 (6.5)</td>
<td>0.27</td>
<td>85.40 (7.5)</td>
<td>81.0 (5.3)</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin (mU/L)</strong></td>
<td>22.3 (14.4)</td>
<td>26.2 (21.6)</td>
<td>0.51</td>
<td>21.9 (14.4)</td>
<td>20.7 (12.7)</td>
<td>0.78</td>
<td></td>
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<tr>
<td><strong>HOMA-IR</strong></td>
<td>4.5 (3.0)</td>
<td>5.1 (3.9)</td>
<td>0.64</td>
<td>4.6 (3.1)</td>
<td>4.1 (2.4)</td>
<td>0.53</td>
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</tbody>
</table>

Except for sex (actual patient numbers), data are shown with mean ± standard deviation (SD). p<0.05 was considered significant. AST = aspartate aminotransferase; ALT = alanine aminotransferase; HDL = high-density lipoprotein; LDL = low-density lipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance.
Figure Legends

**Figure 1: Total BA values in treatment vs. placebo groups.** Box plot shows the mean effective serum levels of BA in the placebo and treatment groups. Horizontal line in each box shows mean and top and bottom lines of boxes show interquartile range (IQR). Circles represent outliers.

**Figure 2. FGF19 values in treatment vs. placebo groups.** Box plot shows the mean effective plasma levels of FGF19 in the placebo and treatment groups. Horizontal line in each box shows mean and top and bottom lines of boxes show interquartile range (IQR). Circles represent outliers.* = \( p < 0.01 \).