SOMATO PUBLICATIONS

ISSN: 2641-2292

Journal of Clinical Nutrition and Food Science

Research Article

A New Combination of 2 Nutraceuticals and 1 Probiotic (Epatrex) for Non-Alcoholic Fatty Liver Disease (NAFLD): Results from a Multi-Centre, Randomized, Single-Blind, Parallel, Cross-Over Study: the "REVEAL" Study

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Received: 04 July 2020; Accepted: 01 September 2020; Published: 03 September 2020

Citation of this article: Bellentani, S., Murzilli, S., Vanelli, A. (2020) A New Combination of 2 Nutraceuticals and 1 Probiotic (Epatrex) for Non-Alcoholic Fatty Liver Disease (NAFLD): Results from a Multi-Centre, Randomized, Single-Blind, Parallel, Cross-Over Study: the "REVEAL" Study. J Clin Nutr Food Sci, 3(2): 01-13.

Abstract

No pharmaceutical or nutraceutical treatments for non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) are currently available on the market, although there are different molecules in clinical trials (Phases 2 and 3). All guidelines suggest only lifestyle changes. In this article, the authors report a single-blind cross-over pilot study of the effect of a new nutraceutical, Epatrex. The primary outcomes were the in vivo safety and effects on gamma glutamyl transferase (GGT) levels and fatty liver index (FLI) values of a new 3-layer tablet containing the active ingredients. After 2 months of treatment with Epatrex or the placebo, the new product was found to be safe, although no significant differences in the absolute values of the fatty liver index (FLI) or GGT and triglyceride (TG) levels were observed. However, the percentage reductions in GGT and TG levels in patients treated with Epatrex were significantly greater ($p<0.005$) than those in patients treated with the placebo at 60, 90 and 150 days after the beginning of the study. Several studies have reported microbiota changes involving Lactobacillus Casei after 1- 3 weeks of daily administration, and for this reason we chose 8 weeks of treatment to obtain clinical results. We concluded that 2 months of treatment with Epatrex, a three-layer nutraceutical composed of Lactobacillus casei LC-XCAL + silymarin/silybin + chromium picolinate, was safe, albeit unable to significantly improve the FLI or GGT values; the reductions in the GGT and TG values were not significant. More studies focusing on

different doses and treatment periods are needed to confirm that Epatrex is a useful treatment for NAFLD.

Keywords: Probiotics, NAFLD, Reveal study, Epatrex

Introduction

Hepatic steatosis is a very common disease throughout the Western world. The prevalence in Italy is approximately 35-40% in the general population [1-3]. The most common aetiologies are usually excessive alcohol consumption [(this is the case in alcoholic liver steatosis (AFLD)] and so-called dysmetabolic causes, which are grouped together as non-alcoholic fatty liver disease (NAFLD). NAFLD includes a wide range of diseases, including dyslipidaemia, diabetes, obesity, and metabolic syndrome (MS), all of which involve insulin resistance (IR). NAFLD is considered the hepatic manifestation of MS [4]. NAFLD is a highly prevalent reversible clinical condition affecting 25% of the general adult population [5-7] and approximately 15% of children, and it is already the second leading cause of liver transplantation in the USA [8]. The prevalence is twice as high in obese and diabetic patients. Almost 1/3 of NAFLD cases may evolve to a more aggressive form, namely, non-alcoholic steatohepatitis (NASH), which is characterized by inflammation and progressive tissue degeneration and affects approximately 5% of the general adult population and 20% of obese people. In 5 years, it is expected that NAFLD, together with NASH, the "active" and progressive form of NAFLD, will be the most common liver disease [9]. The main risk factors for primary NAFLD and NASH are often related to poor lifestyle habits, which can be accompanied by co-morbidities such as overweight/obesity, insulin resistance/type 2 diabetes, hypertriglyceridemia, hypercholesterolemia (all of which are also linked to the excessive consumption of sugar-sweetened soft drinks, mainly containing fructose) [10,11], nocturnal sleep apnoea [12], hypothyroidism [13], cardiovascular diseases [14], extra-hepatic cancer (breast and colorectal) [15], and chronic kidney disease [16]. NASH can also evolve into cirrhosis or hepatocellular carcinoma (HCC) [17,18].

There are currently no pharmaceutical or nutraceutical treatments for NAFLD or NASH, although there are different molecules undergoing clinical trials (Phase 2 and 3), and all available guidelines suggest only lifestyle changes [19-22]. These changes can theoretically be accomplished through multi-disciplinary interventions involving subjects' diet and physical activity and cognitive-behavioural psychotherapy [23]. The level of compliance with these recommendations regarding lifestyle changes is usually very low [24], but all the drugs that are currently used for NASH are still being investigated in more than 200 multi-centre controlled clinical trials, and their prescription is off-label.

Since the only treatments recommended by the major European and US associations (EASL)/(AASLD) guidelines [20,25,26], to date, involve the modification of lifestyle factors (diet and physical activity), we believed that finding a nutraceutical that could improve the biochemical or score markers of steatosis could help as an adjunct therapy to lifestyle changes [27].

The aim of this pilot study was to evaluate the safety and efficacy of a new nutraceutical compound named "Epatrex" compared with a placebo in a multi-centre, randomized, single-blinded, cross-over study in patients with a diagnosis of NAFLD and a gamma glutamyl transferase (GGT) level that was 1.5-fold higher than the upper limit of normal. The study had an intention to treat design.

Materials and Methods

Epatrex is a new nutraceutical compound formed of three components. It functions as antioxidant acting on the intrahepatic metabolism of lipids and carbohydrates.

Epatrex is formulated as a three-layer tablet that contains *Lactobacillus casei* LC-XCAL + silymarin/silybin + chromium picolinate. *Lactobacillus casei* LC-XCAL contains corn starch that supports the fermentation process of the probiotic, but also reduces the free water activity and preserves the vitality of the probiotic. The detailed composition of the layers in both the Epatrex and placebo tablets is reported in Table 1.

The decision to try a new three-layer nutraceutical was based on fairly strong assumptions emerging from the analysis of the mechanisms of action of the hepatoprotective nutraceuticals that are currently available. These are summarized here:

1. LC-XCAL has been shown to reduce triglyceride levels both in animal models and humans. Several studies have reported microbiota changes involving *L. casei* after 1- 3 weeks of daily administration [28-33].
2. Silymarin/silymin acts as an antioxidant on beta-oxidation and as an anti-fibrotic agent in humans. To our knowledge, no other point in metabolism is affected [34,35].
3. Chromium picolinate works by increasing cellular glucose absorption. It improves and reduces IR and lipid metabolism, reducing the triglyceride value. To date, few human studies have been performed; nevertheless, its clear action on liver metabolism and IR are novel [35-38].

Ethical issues and informed consent

The study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of Good Clinical Practice (GCP) of the European Community, CPMP (CPMP/ICH/135/195; ICH Topic E6). The study was conducted in 5 gastroenterology medical centres in Romania in the following cities: Bucharest, Oradea, Iasi, Cluj and Napoca (Table 2). According to the local regulations, studies with food supplements do not need approval from the Romanian Drug Agency or the National Ethics Committee, which strictly regulate drugs and medical devices only.

This study was approved by each ethics committee of the hospitals involved in the study. Approval from all the ethics committees of the hospitals was received prior to starting the study.

All subjects provided written informed consent to participate in the study prior to being screened. The subject information sheet detailed the procedures involved in the study (aims,

Table 1: Placebo and Epatrex tablet composition.

	Ingredients	Intake in Tablet (mg)
Placebo		
1st layer	Microcrystalline cellulose	Q.S.
	Silicon dioxide	Q.S.
	Magnesium stearate	Q.S.
	Dicalcium phosphate	Q.S.
	Cross-linked sodium carboxymethyl cellulose	Q.S.
	E172	Q.S.
2nd layer	Dicalcium phosphate	Q.S.
	Polyvinylpolypyrrolidone	Q.S.
	Corn starch dried	Q.S.
	Hydroxypropylcellulose	Q.S.
	Glyceryl behenate	Q.S.
	Magnesium stearate	Q.S.
	Silicon dioxide	Q.S.
	Microcrystalline cellulose	Q.S.
3rd layer	Microcrystalline cellulose	Q.S.
	Silicon dioxide	Q.S.
	Magnesium stearate	Q.S.
	Cross-linked sodium carboxymethyl cellulose	Q.S.
	E172	Q.S.
Total		1200

Epatrex		
1st layer	Milk thistle	Silybin 90 mg
	Microcrystalline cellulose	Q.S.
	Silicon dioxide	Q.S.
	Magnesium stearate	Q.S.
	Dicalcium phosphate	Q.S.
	Cross-linked sodium carboxymethyl cellulose	Q.S.
	E172	Q.S.
2nd layer	Lactobacillus casei	1×10^9 CFU (at the end of shelf life)
	Dicalcium phosphate	Q.S.
	Polyvinylpolypyrrolidone	Q.S.
	Corn starch dried	Q.S.
	Hydroxypropylcellulose	Q.S.
	Glyceryl behenate	Q.S.
	Magnesium stearate	Q.S.
	Silicon dioxide	Q.S.
	Microcrystalline cellulose	Q.S.
3rd layer	Chromium picolinate	100 mcg (250% NVR)
	Microcrystalline cellulose	Q.S.
	Silicon dioxide	Q.S.
	Magnesium stearate	Q.S.
	Cross-linked sodium carboxymethyl cellulose	Q.S.
	E172	Q.S.
Total		1200

methodology, potential risks, anticipated benefits), and the investigator explained these issues to each subject. Each subject signed the consent form to indicate that the information had been explained and understood. Each subject was given a copy of the informed consent form for their information. One original copy of the informed consent form was kept in a confidential file in the investigators' records.

Sample population

In a cross-over 2 × 2 design, a total sample of 72 subjects was randomized 1:1 to the following sequence of treatment: "Epatrex vs. placebo" and "placebo vs. Epatrex". This ensured a power of 80% to detect a difference in the mean GGT values (primary endpoint) of 20 between the 2 treatment groups. This calculation was based on the assumption of an estimate of the standard deviation of the differences equal to 60 and a significance level equal to 0.05 with a 2-tailed t-test used to analyse paired data. A 10% dropout rate was expected in each arm, and they were replaced to ensure 72 participants per group for the final statistical analysis.

During the study period, the participants received Epatrex (2 cps/day) or the placebo (2 cps per day), based on a computer-generated randomization scheme in a 1:1 allocation model followed by a wash-out period and cross-over. The treatment was administered orally after meals for 60 consecutive days. After 60 days, subjects went through a wash-out period of 30 days. This wash-out period was followed by cross-over, when subjects received the other treatment for another 60 days.

When enrolling the patients, we applied the following inclusion and exclusion criteria.

Table 2: List of investigators, with the starting (SE) and the first subject enrolled (FE) date.

No.	Site	Investigator Name	Approval Date	SE (Date)	FE (Date)
1	SC Gastromedica SRL, Iasi	Anca Trifan, MD, PhD	12.10.2018	16.10.2019	02.11.2018
2	Municipal Hospital Dr. Gavril Curteanu, Oradea, Department of Internal Medicine	Ovidiu Burta, MD, Assoc. Prof	27.10.2018	17.10.2018	06.11.2018
3	"Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology	HoriaStefanescu, MD	01.11.2018	18.10.2018	29.10.2018
4	University Emergency Hospital Bucharest, Department of Internal Medicine	Nicoleta Tiuca, MD	19.10.2018	19.10.2018	25.10.2018
5	Endodigest Medical Clinic, Oradea	Diana Petrisor, MD	15.11.2018	19.11.2018	27.11.2018

Inclusion criteria

- Age 18 - 75 years;
- Signed informed consent provided;
- Confirmed diagnosis of NAFLD grade 1 - 4 by ultrasound (hepatic ultrasound performed a maximum of 3 months prior to screening); ALT or AST > 40 UI/L or GGT > 35 UI/L during the screening period; alcohol intake < 20 g per day or 140 g per week; other causes of liver disease were excluded.

Exclusion criteria

- Alcohol consumption more than 20 g/day or 140 g per week;
- Use of other nutraceuticals within 30 days prior to screening;
- Use of other antioxidant products, including vitamin E, vitamin C, glutathione or probiotics, 30 days before screening;
- Initiation or discontinuation of a dietary regimen 90 days before screening;
- Use of pentoxifylline or gemfibrozil 30 days before screening;
- Allergy or intolerance to silymarin or milk thistle extracts;
- Body mass index (BMI) ≥40;
- Pregnancy or breastfeeding;
- Clinical evidence of current acute or chronic viral hepatitis;
- Clinical signs of biliary tract obstruction;
- Radiographic imaging compatible with cirrhosis or portal hypertension or previous liver biopsies in which the presence of cirrhosis was shown;
- Type II diabetes with oral treatment (with metformin exclusion) such as thiazolidinediones (pioglitazone), alpha-glucosidase inhibitors, exenatide, etc.;
- Poorly controlled diabetes mellitus, assessed with the presence of HbA1c> 8% (or 64 mmol/mol) in the screening period;
- Type I diabetes;
- Evidence of liver decompensation, defined according to any of the following criteria: serum albumin <3 g/dl, total bilirubin> 2 mg/dl, PT/INR> 1.3 times the standard;
- Renal insufficiency (creatinine ≥ 2 mg/dL in the screening phase);
- Hypertension that is not well controlled by current treatments;
- The presence of primary or secondary malignant tumour types;
- The presence of chronic immune-mediated diseases.

Study design

As shown in Table 3, all the subjects attended 4 visits on site during which different activities were performed:

- Visit 1 – baseline visit (day 0) and laboratory investigations;
- Visit 2 – after 60 days of treatment (day 60);
- Visit 3 – cross-over at the end of wash-out 30 days after completing treatment (day 90);
- Visit 4 – end of study after 60 days of the second-line allocation (day 150).
- After applying the inclusion and exclusion criteria, alcohol

Table 3: Scheme of Activities Performed.

Study Activities	Visit 1 (Day 0)	Visit 2 (Day 60)	Visit 3 (Day 90)	Visit 4 (Day 150)
Demographic data and informed consent collection and treatment randomization	X			
Physical measurements (G, BMI, waist circumference, blood pressure, pulse, temperature, respiration rate)	X	X	X	X
Cross-over visit			X	
Laboratory exams (ALT, AST, GGT, serum triglycerides), calculation of FLI [39] and NAFLD fibrosis score [40], and liver sonography	X	X	X	X
Treatment adherence evaluation (drug accountability)		X		X
Determination of adverse events (AE) and need for rescue medication*		X	X	X

consumption in enrolled patients with NAFLD was tested by a guided questionnaire containing questions that explore not only the quantity of alcohol consumed (according to definition by exclusion criteria: lower than 2-3 drinks a day) but also the frequency (monthly or weekly or occasional) and the age of the onset of consumption. For all the patients enrolled, we collected an accurate clinical history, and we performed 4 visits measuring BMI and waist circumference (WC) at baseline and after 150 days. As reported above and in Table 2, we collected blood at baseline and at every visit to check for liver enzymes, lipid and glucose profile, haemochrome, platelet counts, etc. We calculated the Fatty Liver Index (FLI) [37] and NAFLD fibrosis score [38], and we performed hepatic ultrasonography at baseline and at the last visit.

- No restriction or modification in the diet or lifestyle (including physical activities) was suggested to any of the patients enrolled.

Statistical analysis

Three sensitivity analyses were performed. The first two (unadjusted and adjusted using baseline as covariate) were carried out with the original values measured at the end of each treatment period as response. The third analysis (adjusted using change from baseline) was carried out using the absolute change from baseline to the end of the treatment period as the response [41].

The statistical analyses were performed using a generalized estimating equations (GEE) model for a 2-sequence, 2-period, 2-treatment cross-over design with identity link function, normal distribution and a compound symmetry working correlation matrix to take into account repeated measures within patients. The results are reported as least square means (LSMs) with associated two-tailed 95% confidence intervals and corresponding two-sided p-values [42].

Results

Patient enrolment and demographic data

A total of 76 subjects were screened and provided a signed consent form prior to any study assessment. Two [2] subjects were discharged because they did not meet the eligibility criteria. A total of 74 subjects were enrolled and randomized in this clinical study.

The main baseline demographic data collected from the enrolled patients with NAFLD (51 males and 23 females) are reported in Table 4. There were no differences in age, ethnicity, BMI, WC, blood pressure, daily alcohol intake or any of the other parameters measured between the Epatrex and placebo groups.

Only 4 subjects experienced mild adverse events during the study. All adverse events occurred in the first treatment period after baseline, and all adverse events (2 patents had headache, 1 patient had hyperglycaemia and 1 had rhinitis) were definitely not connected with the therapy, showing that the product was safe.

In Table 5, the main liver enzyme levels in the serum of patients treated with Epatrex or the placebo at different times pooled together are reported as LS means of absolute values and LS mean

Table 4: Baseline demographic, physical and alcohol intake features of the treated and placebo groups.

	Epatrex N=37	Placebo N=37	p-value
Age (years)			
Mean ± SD	49.08 ± 12.94	53.08 ± 11.59	
Median (min-max)	50 (26–71)	56 (28–74)	0.1657
Body mass index (BMI)			
Mean ± SD	29.49 ± 4.43	29.72 ± 3.73	
Median (min-max)	28.73 (18.2–39.2)	29.56 (20.9–39.9)	0.8035
Waist circumference (WC; cm)			
Mean ± SD	105.73 ± 12.83	106.73 ± 14.18	0.7514
Median (min-max)	104 (82–138)	107 (69–130)	
Systolic blood pressure (mmHg)			
Mean ± SD	130.24 ± 12.43	131.54 ± 13.61	0.6699
Median (min-max)	130 (109–160)	130 (105–165)	
Diastolic blood pressure (mmHg)			
Mean ± SD	80.57 ± 12.17	78.68 ± 10.97	0.4848
Median (min-max)	80 (60–110)	80 (60–110)	
Alcohol consumers (< 20-30 g/day) [% (n/N)]	45.95% (17/37)	40.54% (15/37)	0.806
Age at first alcohol intake [Mean (min-max)]	18.89% (13-30)	18.63% (12-27)	0.871

*In general, the groups were homogeneous, and randomization did not affect differences in baseline data.

Table 5: The results are reported as LS means and LS mean differences with associated two-tailed 95% CI and p-values. The GEE model for a two-period cross-over was parameterized with fixed effects for treatment and period (models 1 & 3) or with fixed effects for treatment, period and baseline (model 2) and a compound symmetry working correlation matrix. All p-values, LS means, and confidence intervals were calculated from the GEE model.

Endpoint/Analysis Type	**Epatrex LS Mean (95% CI)**	**Placebo LS Mean (95% CI)**	**LS Mean Difference (95% CI)**	**p-Value**
Body mass index				
Unadjusted for baseline values	29.31 (28.39–30.23)	29.38 (28.46–30.3)	-0.07 (-0.25–0.11)	0.4351
Adjusted using baseline as co-variate	29.33 (29.17–29.49)	29.45 (29.29–29.61)	-0.11 (-0.33–0.11)	0.3079
Adjusted using change from baseline	-0.28 (-0.44–-0.11)	-0.17 (-0.34–0.01)	-0.11 (-0.34–0.12)	0.3382

Serum ALT				
Unadjusted for baseline values	41.35 (33.7–49.01)	42.28 (33.81–50.75)	-0.93 (-7.99–6.13)	0.7968
Adjusted using baseline as covariate	41.58 (35.65–47.51)	42.4 (37.45–47.35)	-0.82 (-9.47–7.84)	0.8532
Adjusted using change from baseline	-5.38 (-12.69–1.92)	-4.77 (-10.73–1.2)	-0.62 (-11.14–9.91)	0.9088
Serum AST				
Unadjusted for baseline values	30.1 (26.63–33.58)	33.38 (26.55–40.21)	-3.28 (-9.77–3.22)	0.3227
Adjusted using baseline as covariate	30.99 (28–33.97)	32.61 (29.18–36.03)	-1.62 (-6.23–2.99)	0.4907
Adjusted using change from baseline	-4.43 (-8.51–-0.34)	-3.99 (-8.3–0.32)	-0.44 (-5.48–4.6)	0.8647
Serum GGT				
Unadjusted for baseline values	64.41 (38.1–90.73)	52.06 (40.97–63.16)	12.35 (-9.52–34.22)	0.2685
Adjusted using baseline as covariate	66.68 (51.17–82.19)	50.46 (35.69–65.22)	16.22 (-11.44–43.88)	0.2504
Adjusted using change from baseline	2.16 (-10.7–15.02)	-15.65 (-35.93–4.62)	17.82 (-12.4–48.03)	0.2478
Serum triglycerides				
Unadjusted for baseline values	146.34 (129.74–162.93)	153.66 (136.14–171.18)	-7.32 (-18.32–3.68)	0.1920
Adjusted using baseline as covariate	145.6 (133.2–158)	154.62 (139.68–169.55)	-9.02 (-21.15–3.12)	0.1452
Adjusted using change from baseline	-25.66 (-42.37–-8.95)	-13.45 (-29.34–2.45)	-12.21 (-32.55–8.13)	0.2392
Fatty liver index (FLI)				
Unadjusted for baseline values	71.87 (66.27–77.46)	72.72 (67.17–78.26)	-0.85 (-3.75–2.05)	0.5655
Adjusted using baseline as covariate	72.75 (69.83–75.68)	72.42 (69.39–75.46)	0.33 (-3.81–4.47)	0.8756
Adjusted using change from baseline	-4.51 (-7.47–-1.55)	-4.89 (-7.91–-1.87)	0.37 (-3.86–4.61)	0.8621

Table 6: Percentage of subjects with improved laboratory values during the study with respect to the baseline value (Day 0).All the data are reported as percentages (%) and absolute values (number of patients with improved values with respect to the total number of patients (n/N)). TG = triglycerides.*p< 0.005

	Day 60			Day 90		Day 150	
	End of First-Line Therapy			1st Day of Second-Line Therapy		End of Second-Line Therapy	
	Placebo [%(n/N)]	Epatrex-[%(n/N)]		Placebo [%(n/N)]	Epatrex[%(n/N)]	Placebo [%(n/N)]	Epatrex-[%(n/N)]
Serum AST	30.8 (4/13)	35.7 (5/14)	Crossover	76.9 (10/13)	42.9 (6/14)	84.6 (11/13)	57.1 (8/14)
Serum ALT	42.9 (6/14)	53.8 (7/13)		64.3 (9/14)	53.8 (7/13)	71.4 (10/14)	69.2 (9/13)
Serum GGT	23.5 (4/17)	41.7* (5/12)		47.1 (8/17)	50.0 (6/12)	52.9 (9/17)	91.7* (11/12)
Serum TG	42.1 (8/19)	76.2* (16/21)		42.1 (8/19)	76.2* (16/21)	57.9 (11/19)	85.7* (18/21)

differences with associated two-tailed 95% CI and p-values.

The GEE model for a two-period cross-over was parameterized with fixed effects for treatment and period (models 1 & 3) or with fixed effects for treatment, period and baseline (model 2) and a compound symmetry working correlation matrix. All p-values, LS means, and confidence intervals were calculated from the GEE model.

As evidenced in Table 5, none of the parameters studied were significantly different between patients treated with the placebo and those treated with Epatrex, probably due to the high degree of variability of the data. However, Epatrex therapy reduced the baseline values of triglycerides by 25%, and the difference almost reached statistical significance.

As reported in Table 6, the percentage reduction intransaminase levels in patients treated with Epatrex compared with the levels in patients who received the placebo did not vary according to the time interval, while the percentage reductions in GGT and TG levels in patients treated with Epatrex compared with those in patients treated with the placebo were significantly greater ($p<0.005$) at 60 and 150 days for GGT and always significantly greater for TG (Table 6 and Figure 1).

There was no statistically significant improvement in any of the other parameters measured, including BMI, WC, FLI, in either treatment line at any time point (data not shown).

Discussion and Conclusions

While waiting for an effective active drug treatment for NASH, we are convinced that the prescription of specific supplements or nutraceuticals with demonstrated hepatoprotective action should be considered to accelerate improvement in altered levels of liver enzymes and possibly in liver steatosis or at least to slow down its evolution. This rationale informed the design of this study. We chose a new product named Epatrex, which is a 3-layer tablet with a new probiotic that could ideally act on different steps of hepatic metabolism, as explained in the introduction and shown in Figure 1.

The results are negative because the differences in the absolute values of the laboratory enzymes and other markers of fatty liver and fibrosis between NAFLD patients treated with Epatrex and patients taking the placebo were not statistically significant (Table 4 and 5); significance was statistically relevant only when

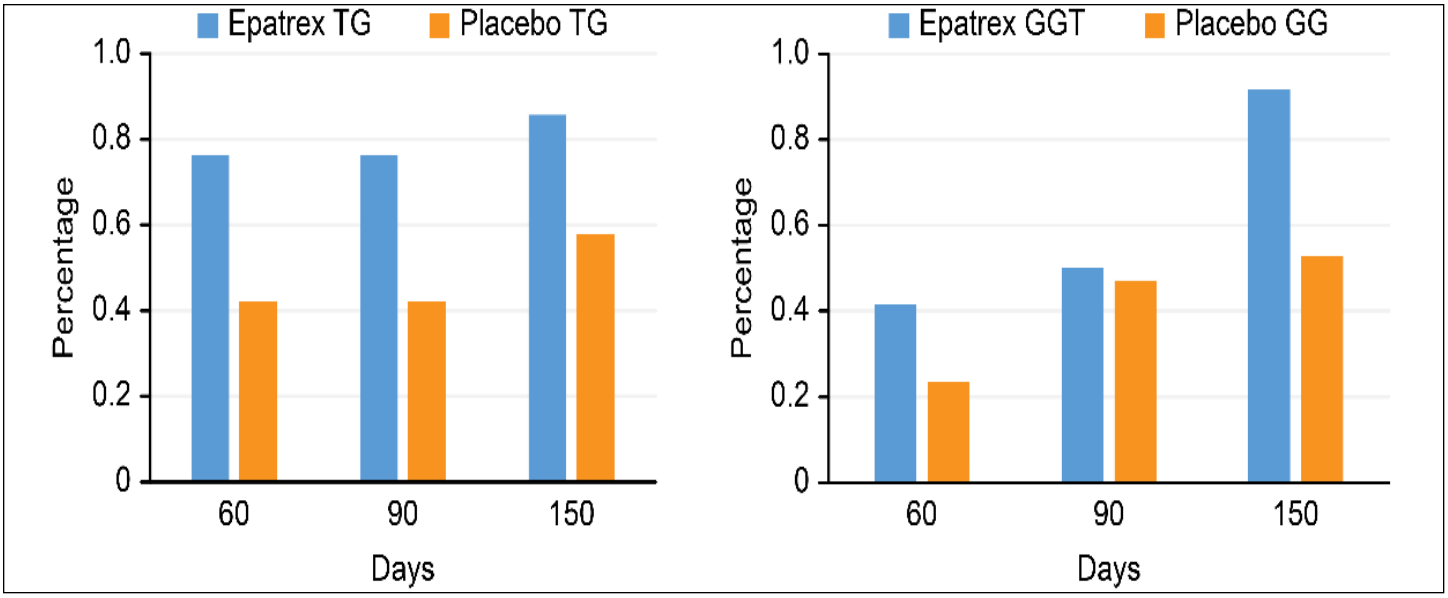

Figure 1: Percentage of patients treated with Epatrex (blue) or the placebo (orange) with reductions in triglycerides (on the left) and gamma glutamyl transferase (GGT) (on the right) at 60, 90 and 150 days.

we measured the percentage of patients who had improved blood levels of GGT and TG (Table 5 and Figure 2). This study has 2 main limitations:

a) The baseline data for patients in both the Epatrex and placebo treatment groups are very heterogenous and have very wide minimum and maximum ranges.

b) Furthermore, the period of treatment for each arm before the cross-over was probably too short to observe a significant difference.

Since the percentages of patients treated with Epatrex who had reduced values of GGT and triglycerides was statistically significantly greater than the percentages of those treated with the placebo, we can speculate that if we had a larger sample size and/or increased the treatment duration, we would probably see significant differences in the raw absolute values.

The severity of the disease describes the clinical status of the participating subjects according to the current diagnostic criteria for NAFLD. The researchers were aware that this was a preliminary study evaluating a new combination product to treat NAFLD-related clinical symptoms, and further research will be necessary to prove this concept on a larger scale.

In conclusion, we think that this pilot study clearly showed that it is possible to develop, according to Evidence-Based Medicine (EBM) rules, a nutraceutical that could be tested in patients with NAFLD. Epatrex, a three-layer nutraceutical composed of *Lactobacillus casei* LC-XCAL + silymarin/silybin + chromium picolinate, was demonstrated to be safe, although it did not prove to be effective for the treatment of NAFLD at the dose and for the duration we used in this pilot study. More studies with rigorous EBM criteria, stratification of the enrolled participants by age, a larger sample size, different doses and different periods of treatment are needed to confirm that Epatrex is a effective treatment for NAFLD.

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Supplementary Material

Table S1: List of abbreviations used in the paper.

Abbreviation	Definition
AE	Adverse event
LSM	Least square mean
IMP	Investigation medical product
NAFLD	Non-alcoholic fatty liver disease
AFLD	Alcoholic fatty liver disease
BMI	Body mass index
WC	Waist circumference
FLI	Fatty liver index
MS	Metabolic syndrome
GEE	Generalized estimating equation
AST	Aspartate transferase
ALT	Alanine transferase
GGT	Gamma glutamyl transferase
TG	Triglyceride