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Randomized Controlled Trial

Effects of oleoylethanolamide supplementation on the expression of lipid metabolism-related genes and serum NRG4 levels in patients with non-alcoholic fatty liver disease: A randomized controlled trial

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SUMMARY

Background: This study investigated the effects of oleoylethanolamide (OEA) supplementation on the expression levels of *SIRT1*, *AMPK*, *PGC-1α*, *PPAR-γ*, *CEBP-α* and *CEBP-β* genes and serum neuregulin 4 (NRG4) levels in patients with non-alcoholic fatty liver diseases (NAFLD).

Methods: Sixty obese patients with NAFLD were equally allocated into either OEA or placebo group for 12 weeks. The mRNA expression levels of genes were determined using the reverse transcription polymerase chain reaction (RT-PCR) technique. Serum NRG4 level was also assessed using an enzyme-linked immunosorbent assay (ELISA) kit.

Results: At the endpoint, mRNA expression levels of *SIRT1*($p = 0.001$), *PGC-1α* ($p = 0.011$) and *AMPK* ($p = 0.019$) were significantly higher in the OEA group compared to placebo group. However, no significant differences were observed in the expression levels of *PPAR-γ*, *CEBP-α* and *CEBP-β* between the two groups. Serum NRG4 levels significantly increased in the OEA group compared with the placebo group after controlling for confounders ($p = 0.027$). In the OEA group, significant relationships were found between percent of changes in the expression levels of the *SIRT1*, *AMPK* and *PGC-1α* as well as serum NRG4 level with percent of changes in some anthropometric measures. Moreover, in the intervention group, percent of changes in high-density lipoprotein cholesterol was positively correlated with percent of changes in the expression levels of the *SIRT1* and *AMPK*. While, percent of changes in triglyceride was inversely correlated with percent of changes in the expression levels of *SIRT1*.

Conclusion: OEA could beneficially affect expression levels of some lipid metabolism-related genes and serum NRG4 level.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of hepatic diseases related to metabolic complications such as obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM),

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and dyslipidemia, all of which elevate the risk of developing cardiovascular diseases (CVD) [1–3]. Accumulating evidence suggests that disruption in the liver lipid metabolism contributes to the genesis of steatosis and NAFLD [4–7]. Hepatic uptake and *de novo* lipogenesis are enhanced in NAFLD, whereas enhanced acquisition of fatty acids is not compensated by fatty acid oxidation (FAO), which can stimulate disease progression by compromising mitochondrial function [4,8].

In recent years, an expanding body of research documented that sirtuin 1 (*SIRT1*) has beneficial effects on hepatic lipid metabolism through inhibition of hepatic lipogenesis, induction of FAO, and maintenance of cholesterol/bile acid levels. The disruption of SIRT1 signaling in the liver causes an accumulation of excessive fat and induces inflammatory responses in both animals and humans [9,10]. *SIRT1* promotes fat mobilization in adipocytes by repressing peroxisome proliferator-activated receptor-γ (*PPAR-γ*) [11]. Furthermore, *SIRT1* can exert anti-inflammatory effects and improve insulin sensitivity in adipocytes. *SIRT1* attenuated tumor necrosis factor-α (TNF-α)-induced IR in adipocytes [12]. In the liver, *SIRT1* interacts and deacetylates PPAR-γ-coactivator-1α (*PGC-1α*) to activate gluconeogenic and FAO genes [13]. It has been demonstrated that *SIRT1* deacetylation of *PGC-1α* may be required for the activation of mitochondrial FAO [14]. *PGC-1α* modulates metabolic homeostasis in the liver and regulates mitochondrial function, gluconeogenesis and lipid metabolism. Overexpression of hepatic *PGC-1α* resulted in increased hepatic FAO with decreased triacylglycerol accumulation and secretion *in vivo* and *in vitro* [15]. AMP-activated protein kinase (*AMPK*) has been demonstrated to directly enhance *PGC-1α* protein activity, perhaps by a direct phosphorylation [16,17]. Because *AMPK* plays a key role in metabolic regulation, it has become an attractive target for metabolic disorders such as obesity and NAFLD [18,19]. Activation of *AMPK* is proposed to reduce liver triglyceride (TG) content through suppression of acetyl CoA carboxylase (ACC) to cause inhibition of *de novo* lipogenesis and stimulation of FAO [18,20]. Members of the CCAAT/enhancer-binding protein (CEBP) transcription factor family including *CEBP-α* and *CEBP-β* have also emerged as important regulators of hepatic lipid metabolism [21,22]. Liver-specific disruption of the *CEBP-α* in obese diabetic mice led to a reduction in hepatic TG accumulation and total cholesterol by inhibition of the expression of lipogenic genes [23]. Furthermore, Rahman et al. [24] demonstrated that *CEBP-β* may play a key role in steatosis and the pathophysiology of nonalcoholic steatohepatitis, through its effect on genes involved in lipid synthesis, inflammation, and possibly endoplasmic reticulum stress in mice.

Prior studies have demonstrated that an inadequate brown adipose tissue (BAT) activity can also play an important role in the pathogenesis of excessive intrahepatic TG accumulation. In fact, there is a link between a decreased BAT activity and NAFLD [5,25]. Recent reports indicate that BAT can release regulatory molecules, so-called brown adipokines or batokines, that can target peripheral tissues such as liver [26,27]. Neuregulin 4 (NRG4) has recently been identified as a novel batokine with beneficial metabolic effects [28]. To data, NRG4 can bind to hepatocytes and attenuate hepatic lipogenic response [29,30]. Decreased serum NRG4 level is also reported to be an independent risk factor for NAFLD [31,32], suggesting that NRG4 may have a protective role in the development and progression of NAFLD [28].

Lifestyle modifications including calorie restriction and physical activity interventions are considered as the first-line therapeutic options in NAFLD [33]. Because the cornerstone of treatment in NAFLD is weight reduction [34], the use of novel compounds with anti-obesity effects may be beneficial in the management of these patients. In this context, considerable attention has recently been given to oleoylethanolamide (OEA) as a bioactive lipid amide [35]. OEA has been suggested to be a safe anti-obesity compound [36]. Evidence suggests that OEA, a high-affinity endogenous ligand of peroxisome proliferator-activated receptor alpha (*PPAR-α*), combines with this nuclear transcription factor to stimulate lipolysis and mitochondrial FAO, leading to increased energy expenditure [37,38]. OEA acts in the liver, adipose tissue, and muscle via *PPAR-α* to stimulate FAO, characterized by increased expression of uncoupling proteins 2 (*UCP-2*) [37]. It has been shown that OEA decreases fat production and lipoprotein secretion in hepatocytes through *PPARα*-dependent mechanisms [39].

We previously investigated the effects of OEA along with a low-calorie diet on some of the NAFLD biomarkers such as metabolic parameters, anthropometric indices and the expression of the key genes involved in lipid metabolism and inflammation [40,41]. However, the effects of the combined therapy on some other factors that play important roles in the regulation of lipid metabolism in the liver were not assessed in our previous study or other clinical trials. Therefore, in this study, we examined the effects of OEA supplementation in combination with calorie restriction on the expression of lipid metabolism-related genes including *SIRT1*, *AMPK*, *PGC-1α*, *PPAR-γ*, *CEBP-α*, and *CEBP-β* as well as serum NRG4 levels among obese adult patients with NAFLD.

2. Methods

2.1. Study design

This randomized, triple-blind, placebo-controlled trial is part of previously published study [40–42]. The participants were recruited through advertisements and referrals from physicians and families. The target population was obese adult men and women [body mass index (BMI) between 30 and 40 kg/m^2] aged between 20 and 50 years, with fatty liver on abdominal ultrasonography. An experienced radiologist performed ultrasound examinations at the beginning and end of the study. The severity of liver steatosis was assessed using the ultrasonographic scoring system developed by Hamaguchi et al. [24].

Exclusion criteria were a history of liver cirrhosis or other forms of liver diseases, diabetes mellitus, hypertension, CVD, kidney diseases, polycystic ovary syndrome, thyroid disorders, cancer, or stroke; current use of medications for hyperlipidemia or glycemic control; use of steatogenic medications or corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) during the past year; under calorie-restricted diets or weight loss medications in the past six months; addiction to drinking alcohol; using smoking on a regular basis; pregnancy or lactation; and use of vitamin, mineral, antioxidant, and herbal supplements within the three months prior to the study.

Considering possible loss to follow-up, the minimum sample size of 30 patients per arm was needed based on the mean and standard deviation (SD) of *PPAR-α* reported by a previous study [43] by considering an alpha level of 0.05 and power of 0.8 in two-sided tests.

Before the study, the research protocol was thoroughly explained to all patients, and they provided their written informed consent. Ethical approval for this study was obtained from the Ethics Committee of Research Vice-chancellor of Tabriz University of Medical Sciences (IR.TBZMED.REC.1399.908, Grant number: 69,105) and also registered in the Iranian Registry of Clinical Trials (Trial registration number and date of registration: IRCT20090609002017N32; Registration date: 11/08/2018**,** last update: 15/05/2021).

2.2. Randomization and intervention

Participants were assigned in a 1:1 ratio to each group (OEA or placebo) using block randomization method with a block size of

four, stratified by gender and grading of the severity of NAFLD. Group assignments were blinded to all patients, study investigators, and radiologist until the end of the study. OEA was synthesized in our laboratory (Nutrition Research Center) as previously described [40,43]. Patients in the intervention and placebo groups received 2 × 125 mg OEA and starch capsules daily, respectively, for 12 weeks. According to the previous clinical trial [40,43], this administered dosage of OEA was considered safe with no side effects. Placebo capsules were shaped similarly to OEA capsules. The supplements were delivered every two weeks to the patients and asked to be taken about an hour before main meals (2 capsules per day). The compliance rate was considered ≥90%, and assessed at the end of the trial based on the unused capsules returned by the patients.

Apart from receiving supplements, a low calorie diet was also designed for each patient by an expert dietitian. The Mifflin–St Jeor equation was used to estimate energy requirements of the patients. A personalized calorie-restricted diet, reducing 500 kcal from total energy expenditure, was designed for participants in each group [44]. The percentages of energy derived from carbohydrates, fat, and protein were 55%, 30%, and 15%, respectively. Three-day food records (2 weekdays and a weekend) and weekly phone calls were applied for dietary adherence measurement.

2.3. Measurements

Anthropometric and body composition indices were measured according to the standardized methods at the beginning and at the end of the trial. BMI was calculated by dividing weight (kg) by the square of height (m^2). The Tanita Body Composition Analyzer MC 780 MA was used to measure body composition, including fat mass and muscle mass. To assess physical activity, the International Physical Activity Questionnaire-Short Form (IPAQ-SF) was employed, providing values in metabolic equivalent (MET)-minutes per week [45].

Biochemical measurements were performed on blood samples taken after 12–14 h of overnight fasting. Samples were collected into EDTA tubes, other than for serum NRG4 levels, which were collected in serum separating tubes. Serum NRG4 levels were measured by the enzyme-linked immunosorbent assay (ELISA) kit (Zell bio, Germany).

2.4. Isolation of RNA and the expression measure of genes

Immediately following blood collection, we used whole blood samples in EDTA-treated tubes to isolate peripheral blood mononuclear cells (PBMCs) for assessing the expression levels of the *SIRT1*, *AMPK*, *PGC-1α*, *PPAR-γ*, *CEBP-α*, and *CEBP-β* genes. PBMCs were separated by standard Ficoll density-gradient centrifugation (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) from buffy coats. Total RNA was isolated from freshly obtained PBMCs by ambion Trizol LS reagent (Thermo Fisher Scientific, USA), in accordance with the manufacture's protocol. RNA concentration and purity were assessed using NanoDrop equipment (NanoDrop™ One/One^C, Thermo Scientific, USA). Sample absorbance was measured at 260 nm and 280 nm, and the ratio of the absorbance at 260 and 280 nm (A260/280) was applied to determine RNA purity. The purified total RNA had A_{260}/A_{280} ratio of 1.8–2.1. According to the cDNA synthesis kit protocol, the number 1000 was divided by the RNA concentration obtained from the NanoDrop device. Based on this, the volume of RNA required for synthesis was obtained. RNA integrity was assessed by electrophoresis on 1% (w/v) agarose gel. For synthesis of complementary DNA (cDNA) from single-stranded isolated RNA templates, a mixture of oligo-dT and random hexamer primers and reverse transcriptase were used, based on the manufacturer's protocol (BioFact™ RTase, South Korea).

Real-time polymerase chain reaction (RT-PCR) technique was applied for assessing the mRNA expression levels of genes. The quantitative PCR step was performed using SYBR Green Master mix (South Korea) on a RT-qPCR system (LightCycler® Instrument, Germany). Primer sequences were retrieved from the online Primer Bank database. Sequences of primers used for the human *SIRT1*, *AMPK*, *PGC-1α*, *CEBP-α*, *CEBP-β* and *PPAR-γ* genes are presented in Table 1. The accuracy of designed primers was examined by the anking sequence using Oligo 7 (Rychlik 2007) software. β-actin was used as housekeeping gene for normalizing mRNA levels. To assess fold changes of the expression levels of each gene, the $2^{-\Delta\Delta CT}$ method was applied [46].

2.5. Statistical analysis

The normality of data was checked by Kolmogorov–Smirnov test. The intention-to-treat (ITT) approach was applied for analyzing results. Multiple imputation method was used to impute the missing values. Data for continuous and categorical variables were expressed as means (SD) and percentages, respectively. For comparing baseline characteristics between the two groups, the *chi*-square test and independent samples *t*-test were used, as appropriate. To assess within-group changes, a paired samples *t*-test was used, while an independent samples t-test was performed to examine differences between the groups. Analysis of covariance (ANCOVA) was applied to control for baseline values and confounding variables. To examine the correlations between the percentage of changes in BMI and body composition with the percentage of changes in serum NRG4, as well as the expression levels of the assessed genes, a linear regression analysis was used. IBM SPSS version 23.0 (IBM Crop., Armonk, NY, USA) was applied for statistical analysis. A *p*-value less than 0.05 was considered statistically significant.

3. Results

The study flowchart is presented in Fig. 1. This figure has been previously published [42]. Two patients (One patient in the OEA group and one in the placebo group) did not continue the study because of non-adherence to dietary recommendations. Therefore, out of the 60 participants who met all inclusion/exclusion criteria and enrolled in the study, 58 patients [OEA (n = 29) and placebo (n = 29)] completed the trial. However, 60 participants were included in the final analysis using ITT approach. More than 90% of

Table 1
Sequence of gene primers for qRT-PCR.

SIRT1	Forward TATACCCAGAACATAGACACG
	Reverse CAGCTTCACAGTCAACTT
AMPK	Forward **TGATGACCATGTGCCAAC**
	Reverse **CCCTGATATCTTTGATTGTGG**
PGC-1α	Forward **TGATAAACTGAGCTACCCT**
	Reverse **AAAGGACCTTGAACGAGA**
PPAR-γ	Forward **AAATCTTGACTTGAACGACCA**
	Reverse **CCCTCGGATATGAGAACCC**
CEBP-α	Forward **AACTCACCGCTCCAATGCCTA**
	Reverse **GCTCCCCTCCTTCTCTCATGG**
CEBP-β	Forward **TTTCGAAGTTGATGCAATCGG**
	Reverse **ATCTTTAAGCGATTACTCAGGG**

qRT-PCR, Quantitative real time polymerase chain reaction; *SIRT1*, sirtuin 1; *AMPK*, AMP-activated protein kinase; *PGC-1α*, peroxisome proliferator-activated receptor γ-coactivator-1α; *PPAR-γ*, peroxisome proliferator-activated receptor-γ; *CEBP-α*, CCAAT/enhancer-binding protein-α; *CEBP-β*, CCAAT/enhancer-binding protein-β.

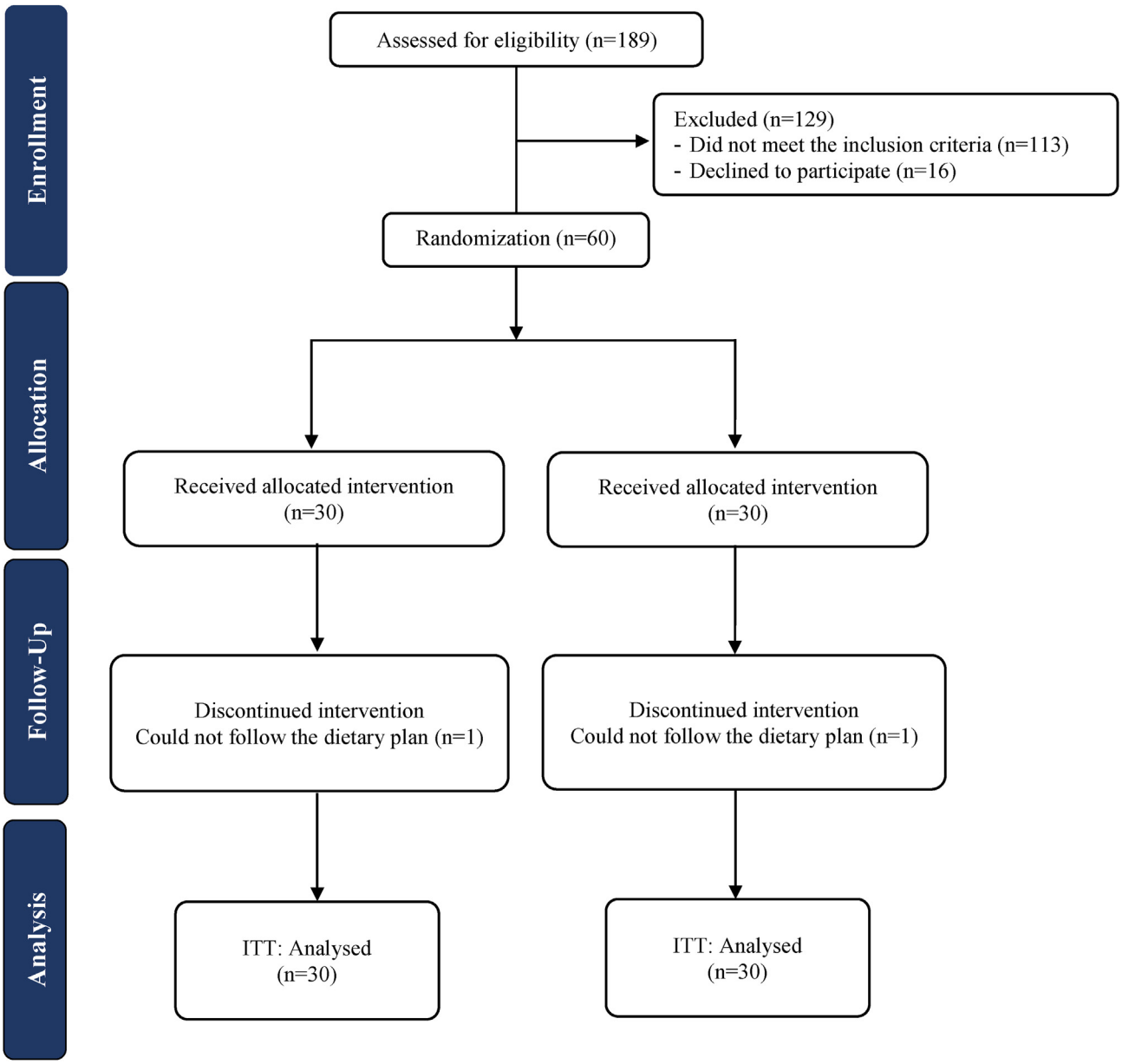

Fig. 1. Study flow diagram. ITT, Intention to treat.

capsules were consumed throughout the study in both groups, demonstrating relatively high compliance. No adverse effects were reported after supplementation. The baseline clinical and demographic characteristics of the two groups were not statistically different with respect to anthropometric data, body composition, energy intake, and physical activity level (Table 2).

After adjusting for baseline values and confounding variables including age, changes in calorie intake, physical activity and BMI, mRNA expression levels of *SIRT1*($p = 0.001$), *PGC-1α* ($p = 0.011$) and *AMPK* ($p = 0.019$) were significantly higher in the OEA supplemented group, compared to their expression levels in the placebo group at the end of the treatment (Fig. 2). No significant differences were observed in the expression levels of *PPAR-γ* (Fig. 3), *CEBP-α* and *CEBP-β* (Fig. 4) between the two groups at the endpoint, though. Possible mechanistic pathways involved in the effects of OEA in the management of NAFLD are shown in Fig. 5.

At the end of the trial, serum NRG4 levels increased significantly in the OEA group when compared with baseline ($p = 0.013$). In addition, a significant increase in serum NRG4 level was found in the OEA group in comparison to the placebo group after controlling for baseline values and potential confounders ($p = 0.027$) (Table 3).

Table 4 provides the correlations between the percentage of changes in BMI and body composition with the percentage of changes in serum NRG4 level, along with the expression levels of the assessed genes in each group. In the intervention group, a significant inverse correlation was seen between percent of changes in BMI with *SIRT1* ($r = -0.394$, $p = 0.031$), *AMPK* ($r = -0.374$, $p = 0.042$) and *PGC-1α* (-0.420, $p = 0.021$) expression levels. Such a significant inverse correlation was seen between

Table 2
Baseline characteristics of the study participants.

	OEA (n = 30)	Placebo (n = 30)	*p*-value
Age (years)	41.78 (9.77)	42.39 (11.77)	0.839[a]
Sex,			
Females	16 (53.34)	16 (53.34)	1.00[b]
Males	14 (46.66)	14 (46.66)	
Weight (kg)	84.32 (13.79)	85.93 (14.77)	0.680[a]
Height (cm)	163.25 (11.19)	164.79 (9.91)	0.585[a]
BMI (kg/m^2)	33.79 (6.69)	34.53 (7.12)	0.692[a]
Energy (kcal/day)	2351.17 (514.61)	2287.39 (429.11)	0.615[a]
FM (%)	36.41 (2.78)	36.98 (1.77)	0.350[a]
FM (kg)	32.69 (3.91)	32.89 (4.66)	0.868[a]
FFM (kg)	56.91 (7.41)	55.33 (6.13)	0.374[a]
Physical activity			
Very low	12 (40.00)	13 (43.33)	0.419[b]
Low	11 (36.66)	9 (30.00)	
Moderate and high	7 (23.34)	8 (26.67)	

BMI, body mass index; FM, fat mass; FFM, fat free mass.
Numerical data are presented as Mean (SD); categorical variables are presented as number (%).
[a] Independent samples t-test.
[b] chi-square test.

percent of changes in serum NRG4 with percent of changes in BMI (r = −0.458, $p = 0.011$). In terms of percentage changes of fat mass, a significant correlation was reached with *SIRT1* (r = −0.436, $p = 0.016$) and *AMPK* (r = −0.420, $p = 0.021$) expression levels in the OEA group. However, positive correlations were observed between percent of changes in fat free mass with the expression levels of *SIRT1* (r = 0.375, $p = 0.041$) and *AMPK* (r = 0.379, $p = 0.039$) in the intervention group. In the placebo group, percent of changes in serum NRG4 was inversely correlated with percent of changes in BMI (r = −0.404, $p = 0.027$) and fat mass (r = −0.375, $p = 0.041$). No significant relationships were found between other parameters in the study groups (Table 4).

In the OEA supplemented group, we found a positive relationship between percent of changes in the expression levels of *SIRT1* (r = 0.523, $p = 0.003$) and *AMPK* (r = 0.381, $p = 0.038$) with percent of changes in high-density lipoprotein cholesterol (HDL-C). Moreover, percent of changes in *SIRT1* expression level was inversely correlated with percent of changes in triglyceride (r = −0.453, $p = 0.012$) in the intervention group. There were no significant relationships between expression of genes and serum levels of

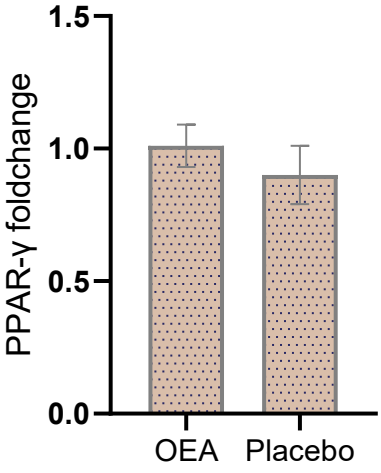

Fig. 3. The effect of intervention on PPAR-γ expression in two study groups: Fold change of PPAR-γ. Values are mean of fold change ± s.e.m. Data analysis was done using One Sample T test (*p < 0.05 vs. baseline) and ANCOVA test (adjusted for age, changes in calorie intake, physical activity and BMI; (**p < 0.05 vs. placebo). p < 0.05 statistically significant. PPAR-γ, peroxisome proliferator-activated receptor-γ.

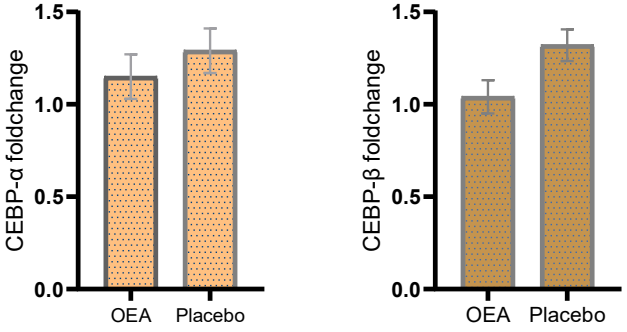

Fig. 4. The effect of intervention on CEBP-α and CEBP-β expression in two study groups: a) Fold change of CEBP-α. b) Fold change of CEBP-β. Values are mean of fold change ± s.e.m. Data analysis was done using One Sample T test (*p < 0.05 vs. baseline) and ANCOVA test (adjusted for age, changes in calorie intake, physical activity and BMI; (**p < 0.05 vs. placebo). p < 0.05 statistically significant. CEBP-α, CCAAT/enhancer-binding protein-α; CEBP-β, CCAAT/enhancer-binding protein- β.

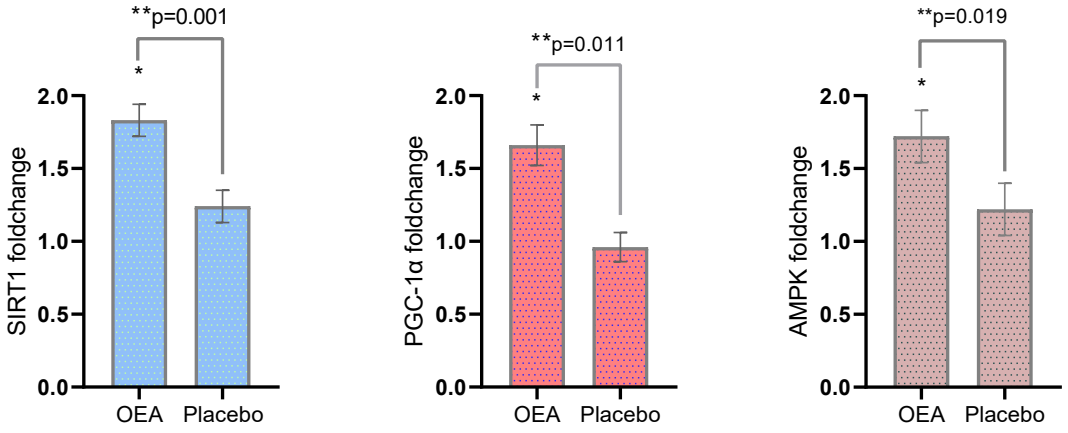

Fig. 2. The effect of intervention on SIRT1, PGC-1α, and AMPK expression in two study groups: a) Fold change of SIRT1. b) Fold change of PGC-1α. c) Fold change of AMPK. Values are mean of fold change ± s.e.m. Data analysis was done using One Sample T test (*p < 0.05 vs. baseline) and ANCOVA test (adjusted for age, changes in calorie intake, physical activity and BMI; (**p < 0.05 vs. placebo). p < 0.05 statistically significant. SIRT1, sirtuin 1; PGC-1α, peroxisome proliferator activated receptor γ-coactivator 1α; AMPK, AMP-activated protein kinase.

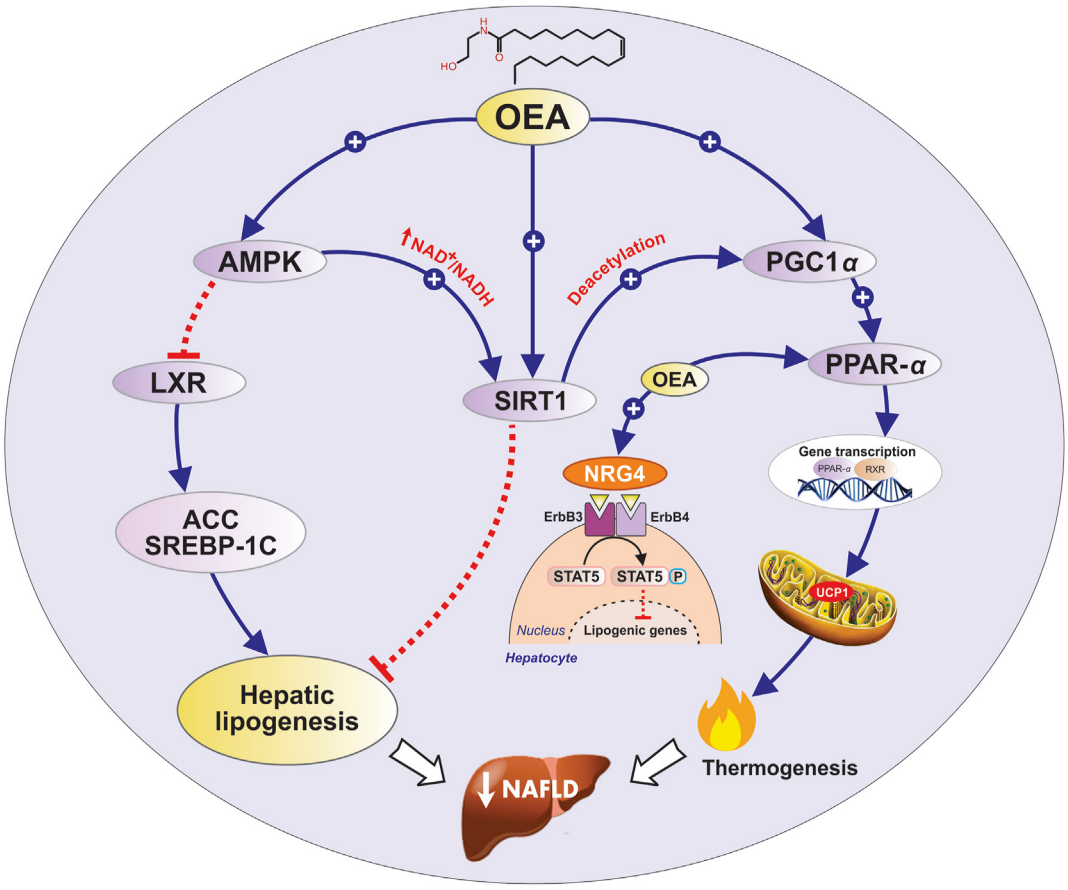

Fig. 5. Possible mechanistic pathways involved in the effects of OEA in the management of NAFLD. ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; LXR, liver x receptor; NAFLD, non-alcoholic fatty liver disease; NRG4, neuregulin 4; OEA, oleoylethanolamide; PGC-1α, peroxisome proliferator activated receptor γ-coactivator 1α; PPAR-α, peroxisome proliferator-activated receptor-α; SIRT1, sirtuin 1; SREBP-1c, sterol regulatory element-binding protein-1c; STAT, signal transducer and activator of transcription; UCP-1, uncoupling protein-1.

Table 3
Serum NRG4 levels of the study participants throughout the study.

	OEA (n = 30)	Placebo (n = 30)	MD (95% CI), *p*-value
NRG-4 (ng/mL)			
Baseline	2.78 (0.59)	3.14 (0.93)	−0.36 (0.62, −1.34), 0.471[b]
End	3.66 (0.96)	3.26 (1.06)	0.53 (0.98, 0.07), **0.027**[c]
MD (95% CI), *P*[a]	0.88 (0.04, 1.72), **0.039**	0.15 (−0.01, 0.31), 0.073	

NRG4, neuregulin 4; OEA, oleoylethanolamide.
Mean (SD) and mean difference (95% CI) are presented for data.
[a] *p*-value based on Paired sample t-test.
[b] *p*-value based on Independent sample t-test.
[c] *p*-value based on ANCOVA adjusted for baseline values, age, changes in physical activity level, energy intake, and BMI.

NRG4 with other metabolic parameters in the study groups (Table 5).

4. Discussion

To our knowledge, this is the first clinical trial investigating the effects of OEA supplementation on the expression levels of *SIRT1*, *AMPK*, *PGC-1α*, *PPAR-γ*, *CEBP-α*, and *CEBP-β* genes as well as serum NRG4 levels in obese patients with NAFLD. OEA, a bioactive lipid mediator, has gained great attention because of its involvement in the regulation of feeding behavior [47]. In addition to controlling food intake, OEA plays a crucial role in regulating peripheral energy balance [36]. Treatment with OEA decreased lipogenesis, and increased lipolysis and FAO in rodent adipocytes and adipose tissue [37]. The findings from in-vitro studies and in-vivo animal models have documented that OEA primarily modulates lipid metabolism by interacting with PPAR-α [37,48]. In fact, administration of OEA increases mRNA expression of *PPAR-α* and several *PPAR-α* target genes involved in different aspects of lipid metabolism [37,48,49]. Our previous results also indicated that OEA significantly enhanced the mRNA expression levels of *PPAR-α* and *PPAR-α* targets including uncoupling proteins 1 and 2 (*UCP1* and *UCP2*) in patients with NAFLD [40].

In the current study, we showed that OEA administration combined with a low-calorie diet could significantly increase mRNA expression levels of *SIRT1*, *AMPK* and *PGC-1α* compared to placebo, in NAFLD patients. Although to our knowledge no prior studies have examined the effects of OEA treatment on the

Table 4
Associations between percent of changes in BMI and body composition with percent of changes in serum NRG4 and expression levels of the genes.

Percent of changes	OEA (n = 30)			Placebo (n = 30)		
	Percent of changes in variables			Percent of changes in variables		
	BMI (kg/m^2)	FM (kg)	FFM (kg)	BMI (kg/m^2)	FM (kg)	FFM (Kg)
SIRT1	−0.394 * (**0.031**)	−0.436 * (**0.016**)	0.375* (**0.041**)	−0.186 (0.326)	−0.230 (0.221)	0.291 (0.119)
AMPK	−0.374 * (**0.042**)	−0.420 * (**0.021**)	0.379 * (**0.039**)	−0.314 (0.091)	−0.187 (0.322)	0.240 (0.201)
PGC-1α	−0.420 * (**0.021**)	−0.322 (0.083)	0.122 (0.521)	−0.320 (0.085)	−0.191 (0.311)	0.299 (0.108)
PPAR-γ	0.096 (0.613)	0.234 (0.213)	0.091 (0.631)	0.091 (0.631)	0.089 (0.641)	0.185 (0.327)
CEBP-α	0.222 (0.239)	0.068 (0.723)	0.224 (0.234)	0.193 (0.307)	0.109 (0.566)	0.140 (0.461)
CEBP-β	0.147 (0.439)	0.045 (0.813)	0.086 (0.651)	0.213 (0.259)	0.046 (0.809)	0.260 (0.166)
NRG4	−0.458 * (**0.011**)	−0.325 (0.08)	0.334 (0.071)	−0.404* (**0.027**)	−0.375* (**0.041**)	0.292 (0.117)

BMI, body mass index; FM, fat mass; FFM, fat free mass. *SIRT1*, sirtuin 1; *AMPK*, AMP-activated protein kinase; *PGC-1α*, peroxisome proliferator activated receptor γ-coactivator 1α; *PPAR-γ*, peroxisome proliferator-activated receptor-γ; *CEBPα* CCAAT/enhancer-binding protein-α; *CEBP-β*, CCAAT/enhancer-binding protein- β; NRG4, neuregulin 4.
Data were normally distributed. *r(*p*) based on the linear regression analysis.

Table 5
Associations between percent of changes in metabolic parameters with percent of changes in serum NRG4 and expression levels of the genes.

Percent of changes	OEA (n = 30)				Placebo (n = 30)			
	Percent of changes in variables				Percent of changes in variables			
	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
SIRT1	−0.171 (0.367)	−0.240 (0.202)	0.523* (**0.003**)	−0.453* (**0.012**)	−0.098 (0.605)	−0.102 (0.592)	0.103 (0.589)	−0.299 (0.108)
AMPK	−0.031 (0.871)	−0.212 (0.261)	0.381 * (**0.038**)	−0.346 (0.061)	−0.232 (0.218)	−0.144 (0.447)	0.050 (0.791)	−0.216 (0.251)
PGC-1α	−0.195 (0.302)	−0.285 (0.127)	0.295 (0.113)	−0.237 (0.207)	−0.026 (0.891)	0.073 (0.701)	0.113 (0.551)	−0.222 (0.238)
PPAR-γ	−0.140 (0.461)	0.116 (0.541)	0.176 (0.353)	−0.121 (0.524)	−0.154 (0.418)	0.059 (0.756)	0.157 (0.406)	−0.216 (0.251)
CEBP-α	−0.047 (0.807)	−0.063 (0.741)	−0.074 (0.699)	−0.042 (0.824)	−0.082 (0.665)	−0.129 (0.497)	−0.137 (0.469)	−0.153 (0.420)
CEBP-β	−0.229 (0.224)	−0.156 (0.409)	0.070 (0.713)	−0.121 (0.524)	−0.026 (0.891)	0.072 (0.707)	0.062 (0.743)	−0.227 (0.228)
NRG4	−0.271 (0.147)	−0.312 (0.093)	−0.303 (0.104)	−0.300 (0.107)	−0.186 (0.324)	−0.192 (0.309)	−0.227 (0.227)	−0.258 (0.169)

TC, Total cholesterol; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol. TG, Triglyceride; *SIRT1,* sirtuin 1*; AMPK*, AMP-activated protein kinase; *PGC-1α*, peroxisome proliferator activated receptor γ-coactivator 1α; *PPAR-γ*, peroxisome proliferator-activated receptor-γ; *CEBPα* CCAAT/enhancer-binding protein-α; *CEBP-β*, CCAAT/enhancer-binding protein- β; NRG4, neuregulin 4.
Data were normally distributed. *r(*p*) based on the linear regression analysis.

expression levels of the aforementioned genes, pharmacological activation of *SIRT1*, *AMPK* and *PGC-1α* has been shown to be effective in the prevention of obesity-related metabolic abnormalities [16]. Previous reports have indicated that *SIRT1* and *AMPK* are important regulators of hepatic lipid metabolism [50]. It has been demonstrated that liver-specific deletion of *SIRT1* or down-regulation of *SIRT1* in mice results in impaired *PPAR-α* signaling, which is related to increased acetylation of *PGC-1α* and leads to reduction of FAO, contributing to the development of liver steatosis [51]. While overexpression of *SIRT1* leads to a decrease in *PGC-1α* acetylation levels and an increase in the expression of *PPAR-α* targets, which results in increased FAO and alleviation of hepatic steatosis [9,51].

In the liver, *AMPK* activation by a number of pharmaceutical agents is proposed to suppress lipid synthesis and promote FAO through phosphorylation and inactivation of ACC. Moreover, the activation of AMPK leads to the reduction in the activity of sterol regulatory element-binding protein-1c (SREBP-1c), which plays a significant role in regulating triglyceride (TG) metabolism. Consequently, stimulating AMPK activation inhibits both ACC and SREBP-1c activity, resulting in reduced intrahepatic lipid accumulation [20,52]. Several studies have provided evidence that SIRT1 expression is downregulated in obesity but upregulated during calorie restriction and weight loss in adipose and other tissues, both in rodents and in humans [16,53]. Obesity is also associated with decreased *AMPK* activation [16]. Moreover, previous studies suggest that hepatic *PGC-1α* expression is downregulated by obesity in human subjects [15]. Collectively, these data indicate that favorable changes in body weight or body composition may exert beneficial effects on the expression levels of the above-mentioned genes.

In terms of OEA group, percent of changes in BMI was inversely correlated with expression levels of *SIRT1*, *AMPK* and *PGC-1α*. Similarly, an inverse correlation was found between percent of changes in fat mass with *SIRT1* and *AMPK* expression levels. However, there was a positive correlation between the percentage of changes in fat free mass and the expression levels of *AMPK* and *SIRT1*. Significant relationships were also found between percent of changes in expression levels of the *SIRT1* and *AMPK* with percent of changes in some metabolic parameters including HDL-C and TG in the OEA group. Weight-lowering effects of OEA have consistently shown in prior studies as well as in our previous results [40, 41, 43, 48]. In addition, previous studies demonstrated that OEA activates *PPAR-α*, which is required to reduce blood lipid levels and enhance peripheral fatty acid catabolism [37, 48]. In a study conducted by Yang et al. [49], it was found that treatment with OEA led to a significant increase in the mRNA expression of fatty acid translocase (FAT/CD36) in the intestinal mucosa and isolated jejunal enterocytes. Moreover, OEA treatment significantly increased fatty acid uptake in isolated enterocytes *in vitro*. The findings suggest that, apart from its role in appetite regulation, OEA also plays a significant regulatory role in lipid metabolism; therefore, it is reasonable to speculate that OEA might enhance the expression levels of the three mentioned genes (*SIRT1*, *AMPK*, and *PGC-1α*) by improving BMI, body composition, and lipid metabolism.

In our study, there was no significant difference in the expression level of *PPAR-γ* between the OEA and placebo groups at the end of trial. Our result aligns with findings from *in vitro* and *in vivo* studies, which have also shown that the biological functions of OEA are mediated by *PPAR-α*, rather than other members of the PPAR family, such as *PPAR-γ* [49].

Evidence from experimental studies indicates that overexpression of *CEBP-α* and *CEBP-β* genes in liver tissue is involved in the development of steatosis and NAFLD by promoting hepatic TG content and inducing the expression of lipogenic genes [21,22]. Therefore, reduced expression levels of *CEBP-α* and *CEBP-β* could have potential benefits for our patients. However, in the current study, we did not observe significant differences in the expression levels of *CEBP-α* and *CEBP-β* genes between the two groups at the end of the trial. It is possible that a higher dosage or longer duration of OEA supplementation might be needed to affect the expression levels of these genes.

Our findings revealed that a 12-week supplementation with OEA, along with a weight loss diet, led to a significant increase in serum levels of NRG4 compared to the placebo group, after controlling for confounding variables. This finding is of particular importance, since decreased serum NRG4 level has been previously reported as an independent risk factor for NAFLD [31,54]. In the OEA group, we also observed a significant inverse relationship between percent of changes in serum NRG4 with percent of changes in BMI. Therefore, the beneficial changes in BMI induced by OEA might be linked to the increased serum NRG4 levels in NAFLD patients. *PPAR* agonists have the potential to stimulate the conversion of white fat to brown fat [55]. BAT releases factors that mediate lipid metabolism and thermogenic activation [26]. NRG4 is one of these factors that has been shown to promote fuel oxidation to ameliorate metabolic disturbances including IR and hepatic steatosis. The liver is considered the target tissue for NRG4, likely due to its direct binding to the ErbB receptors [28]. It has been shown that NRG4 can protect against NAFLD via attenuating hepatic lipogenesis. Mechanistically, NRG4 binds to hepatocytes and triggers the activation of ErbB3 and ErbB4 receptor tyrosine kinases. It subsequently regulates sterol regulatory element binding protein-1c (SREBP-1c) in an inverse manner by trans-repressing the liver X receptor (LXR) [30,56].

The principal strengths of our trial were stratified block randomization design, high level of compliance with treatment protocol, individualized calorie-restricted diet, and inclusion of newly diagnosed NAFLD patients. The use of ITT method and inclusion of all randomized patients enabled us to obtain an unbiased estimation of the intervention effect. The current study had some limitations, as well. We could not examine the effect of OEA on histology of NAFLD, because a liver biopsy was not performed due to ethical problems. We investigated the gene expression in PBMCs rather than liver tissue, which might have biased our measurements. However, this bias is unlikely to differ between study groups. Moreover, available evidence confirms that PBMCs reflect the effects of dietary modifications at the level of gene expression in humans [56]; nevertheless, some over or underestimation of effects might occur. While the study groups were well-balanced for all measured baseline characteristics, differences between the study groups in the distribution of potential unknown confounders cannot be ruled out. In our study, funding restrictions did not allow us to measure serum levels of OEA, which is the most appropriate method for assessing the compliance of the patients. We were also unable to investigate the effect of OEA on study outcomes stratified by gender or other demographic variables because the relatively small sample size made this study underpowered to detect subgroup differences. Moreover, our results cannot be generalizable to older adults and those with severe fatty liver disease. Finally, due to financial constraints, we failed to investigate the effects of supplementation on the expression of other lipid metabolism-related genes. Large-scale studies are needed to address the effects of high-dose and long-term OEA supplementation on study outcomes, stratified by gender or other demographic variables.

5. Conclusion

In conclusion, OEA supplementation accompanied by weight loss diet increased not only the expression levels of *AMPK*, *SIRT1* and *PGC1-α* in PBMCs but also enhanced serum NRG4 levels in patients with NAFLD with no side effects. However, supplementation with OEA could not affect expression levels of *PPAR-γ*, *CEBP-α*, and *CEBP-β* genes.

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Authors' contributions

Helda Tutunchi, Alireza Ostadrahimi, Mehrangiz Ebrahimi-Mameghani, Mohammad Javad Hosseinzadeh-Attar, Majid Mobasei, and Farzad Najafipour contributed to the conception and design of the research. Fatemeh Naeini and Sina Naghshi contributed to data collection and Neda Roshanravan, Samira Asghari, and Moloud Akbarzadeh helped with RT-PCR. Helda Tutunchi drafted the manuscript and analyzed and interpreted the data. Hamid Soleimanzadeh contributed to synthesis of the supplement. All authors critically revised the manuscript, agreed to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final draft of the manuscript for publication.

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