

A randomized placebo-controlled, double-blind study to investigate the effect of a high oral loading dose of cholecalciferol in non-alcoholic fatty liver disease patients, new insights on serum STAT-3 and hepassocin

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Abstract. – OBJECTIVE: Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver metabolic disease affecting millions globally. This study aimed to assess the safety and efficacy of a high oral loading dose of cholecalciferol supplement on NAFLD patients and to investigate its potential role on serum inflammatory biomarkers.

PATIENTS AND METHODS: One hundred patients with NAFLD and type 2 diabetes mellitus were involved in the study. Eligible patients were randomized among two equal groups. Group 1 received the standard conventional therapy in addition to a placebo. Group 2 received the conventional therapy plus cholecalciferol for 4 months. The improvement in the patients' glycaemic control parameters, liver function tests, lipid profile, and serum 25-hydroxy vitamin D at the end of the study was set as a primary outcome. The secondary outcome was the decrease in steatosis grade in the ultrasound and high-sensitivity C-reactive protein (hs-CRP), tu-

mor necrosis factor-alpha (TNF- α), signal transducer and activator of factor-3 (STAT-3), nitric oxide (NO), malondialdehyde (MDA), and hepassocin serum levels at the end of the study.

RESULTS: Group 2 revealed a significant reduction in the serum levels of lipid profile measures, hs-CRP, alanine aminotransferase (ALT), STAT-3, NO, hepassocin, and MDA compared to the baseline and group 1 results. Whereas group 1 did not show these significant changes. Both groups observed no significant changes in glycaemic index, TNF- α , aspartate aminotransferase (AST), and albumin levels.

CONCLUSIONS: Cholecalciferol is recommended as additional therapy to modulate lipid peroxidation and systemic inflammation alongside other NAFLD therapies.

Key Words:

Non-alcoholic fatty liver, Cholecalciferol, Hepassocin, STAT-3, hs-CRP.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is widely regarded as the leading cause of chronic liver disease and a major public health concern worldwide. The incidence of NAFLD, closely related to obesity, is believed to be 24% worldwide, causing a significant clinical and financial burden on many nations, including the United Kingdom^{1,2}. NAFLD, a broad term embracing a variety of histopathologies from hepatic steatosis (non-alcoholic fatty liver, NAFL) through non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, is defined physiologically by excess lipid buildup in the liver. The disease's phenotype and its progress vary greatly between individuals and are affected by complex interactions between genetic, metabolic, and environmental factors^{3,4}.

Due to the excessive fat accumulation in this disease, NAFLD affects more than 70% of people with metabolic syndrome (MS)⁵. The combination of numerous factors, including environmental, genetic, hormonal, and dietary factors, is the primary cause of NAFLD. The most frequent risk factors for NAFLD beginning are obesity and MS, which are also associated with a faster rate of disease development⁶.

Until now, weight loss and lifestyle change have been the mainstay of treatment for NAFLD. According to guidelines⁷, no approved pharmacological therapy exists, and many patients do not respond to non-pharmacological interventions alone. As a result, numerous medications for treating NAFLD with various mechanisms of action are gaining great interest and are currently undergoing clinical investigation⁸.

Different dietary elements may have different effects on the onset and/or progression of the disease and have been linked to the pathophysiology of NAFLD. For instance, dietary macronutrients appear to variably affect lipid building up in the liver through several molecular and cellular pathways, apart from their role in energy production^{9,10}. Similarly, how micronutrient deficiencies have been linked to NAFLD and their therapeutic targeting has a mechanistic explanation. Unfortunately, nutritional supplements have only been the target of limited intervention trials in individuals with NAFLD, with various results¹¹. In pre-clinical animals, vitamin D has been demonstrated to have anti-proliferative, anti-inflammatory, and anti-fibrotic characteristics that have been proven to slow the worsening of NAFLD^{12,13}.

Moreover, pediatric NAFLD is a notable example of the prevalent poor vitamin D status and low dietary vitamin D intake^{14,15}. However, vitamin D supplementation intervention trials in NAFLD patients have varied durations, dosages, and outcome measures. There are still concerns about the best regimens for treating chronic liver disease¹⁶. Various clinical trials¹⁷⁻¹⁹ have demonstrated the correlation between NAFLD and low vitamin D levels. Moreover, it correlates with the degree of fibrosis and inflammation in NAFLD.

Hepassocin (HPS) is a hepatokine involved in liver regeneration that is produced in hepatoma cells *in vitro* by the hepatocyte nuclear factor 1 (HNF1)-regulated IL-6/IL-6R/STAT3 pathway²⁰. HPS was detected in high serum levels in mice and patients with NAFLD, and HPS levels were linked to non-alcoholic steatohepatitis²¹. In mice, overexpression of hepatic HPS-induced lipid building up by an extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent pathway²². In HPS knock-out mice, HPS is also attributed to the development of IR and type 2 diabetes mellitus (T2DM) through ERK1/2 activation²³.

The current study aimed to investigate the effect of a high oral loading dose of vitamin D on glycaemic parameters, lipid profile, and oxidative and inflammatory biomarkers.

Patients and Methods

This research was conducted at Tanta University's Hepatology Department, Faculty of Medicine, from October 2022 to February 2023. The study included 100 outpatient clinic patients who met the inclusion criteria. Tanta University Faculty of Medicine's National Research Ethics Committee approved this study with approval code (35927/10/2022). The study's design and methods were in accordance with the Helsinki Declaration in 1964 and its later amendments. Patients were informed that they could withdraw from the study at any moment. Trial registration identifier: NCT05578404.

Inclusion Criteria

- Male or female adult patients (> 19 years).
- Fatty liver patients diagnosed by using upper abdominal ultrasound echography (US) and with T2DM diagnosed according to American Diabetes Association (ADA) 2019 criteria and treated with metformin²⁴.

Exclusion Criteria

- Pregnant and/or lactating women.
- Excessive alcohol use (Consistent with the national definition, excessive alcohol consumption was defined as binge drinking (≥ 4 drinks per occasion for a woman and ≥ 5 drinks per occasion for a man)²⁵.
- Chronic liver diseases such as viral, drug-induced, and autoimmune hepatitis.
- Patients suffering from chronic kidney disease.
- Patients with thyroid disorders.
- Hypersensitivity to cholecalciferol.
- Patients taking vitamin D supplementation and medications affecting calcium/vitamin D metabolism.
- Inflammatory bowel disease patients taking systemic steroids or biological therapy.
- Patients already taking anti-inflammatory drugs or other therapies that may affect the serum levels of the measured parameters.

Study Intervention

This was a randomized, controlled, double-blind clinical study that investigated the safety and efficacy of cholecalciferol in the treatment of NAFLD patients and was conducted at Tanta University’s Hepatology Department. This research was registered as NCT05578404 on WWW.ClinicalTrials.gov in 2022. According to the CONSORT flow diagram in Figure 1, the patients were randomly assigned to groups 1 or

2 (n = 50) based on the days of hospitalization. The study included 150 patients who were screened. Forty patients were excluded from the study; 35 could not meet the inclusion criteria outlined above, and 15 declined to participate. One hundred patients continued and were involved in the study and randomized into one of the following two groups:

- Group 1: 50 patients received the standard conventional therapy plus a placebo for 4 months.
- Group 2: 50 patients were given the standard conventional therapy plus cholecalciferol. Cholecalciferol was given at a high oral loading dose of 300,000 IU (Devit^R 300,000 IU, Ampoule, Deva Pharma, Istanbul, Turkey), followed by a daily oral dose of 800 IU IU (Vi-drop^R, Oral Drops, Medical Union Pharmaceuticals company, Nasr City, Egypt) for 4 months. This dose was given to all 50 participants irrespective of their initial 25(OH)D levels.

Regular exercise in the form of any physical activity, such as walking, cycling, etc., for 30-45 minutes at a minimum five days a week, as well as calorie restriction in overweight and obese patients (1,200-1,500 and 1,000-1,200 kcal/day for men and women, respectively), were all components of the standard conventional therapy in both groups. To guarantee the correct treatment assignment, study drugs were administered to patients by an unblinded pharmacist; however, this pharmacist was excluded from the outcome eval-

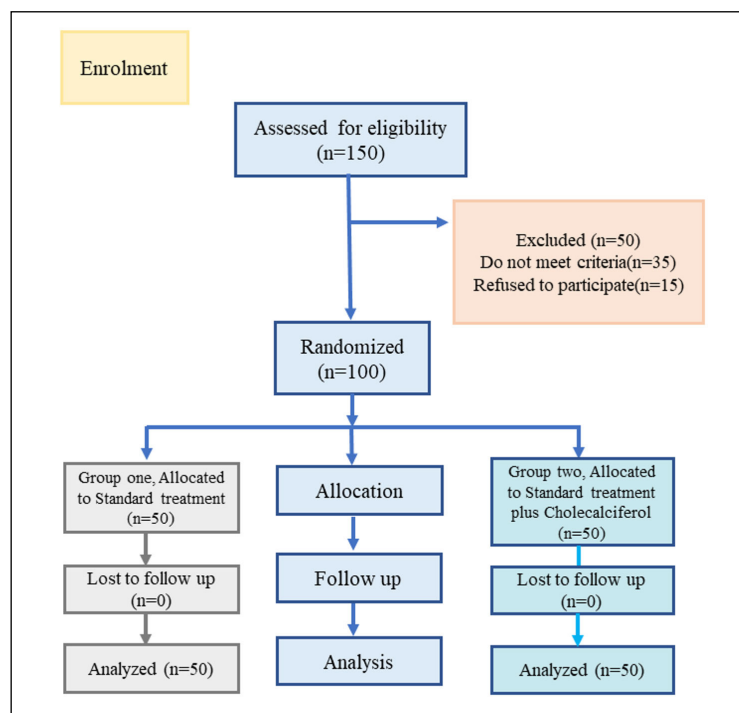


Figure 1. CONSORT flow diagram of patient allocation.

uation. Based on the abdominal US performed by a blinded radiologist, where the liver brightness and liver parenchyma with diffuse echogenicity confirm the diagnosis, all patients were determined to have NAFLD.

Study Procedures

Age, sex, and weight were among the details that the patients' information was collected. Based on the results of liver brightness, deep attenuation of the US signal, hepatorenal echo contrast, and the blurring of vessels, the fatty liver was graded as none (0), mild (1), moderate (2), or severe (3)²⁶. The grading was recorded two times; the first time at the beginning of the study and the second time after 4 months.

Laboratory Measurements and Clinical Assessments

Anthropometric measurements of each patient, including height in meters, weight in kilograms, and waist circumference (WC) in centimeters, were measured at the start and end of the trial (measured midway between the 12th rib and the iliac crest). Moreover, the body mass index (BMI) was determined (BMI = weight in kg divided by height in m²). The World Health Organization (WHO) defines overweight as having a BMI of more than 25 kg/m², and obesity as having a BMI greater than 30 kg/m². Patients were regarded to be vitamin D deficient when their 25(OH)D levels were less than 20, insufficient when they were between 21 and 29, and sufficient or normal when they were at 30 ng/ml or more²⁸.

Serum samples were collected from each patient and used to measure biochemical markers by Enzyme-Linked Immunosorbent Assay (ELISA) using widely available ELISA kits according to the manufacturer's instructions (Sunredio, Shanghai, China) for measuring serum STAT-3 (Kit Catalogue No.: 201-12-0651), serum TNF- α (Kit Catalogue No.: 201-12-0083), MD (Kit Catalogue No.: 201-12-1372), NO (Kit Catalogue No.: 201-12-1511), hepassocin (Kit Catalogue No.: 201-12-1547), hs-CRP (Kit Catalogue No.: 201-12-1806), cholecalciferol (Kit Catalogue No.: 201-12-1547), albumin (Kit Catalogue No.: 201-12-1806), and insulin (kit Catalogue No.: 201-12-0011).

Enzymatic colorimetric techniques were also used to quantify the following laboratory tests: triglycerides (TG mg/dl), total cholesterol (TC mg/dl), low-density lipoprotein (LDL mg/dl), and high-density lipoprotein (HDL mg/dl), alanine and aspartate transaminases (ALT and AST, re-

spectively), fasting blood glucose (FBG mg/dl).

Insulin resistance was calculated by the homeostasis model assessment insulin resistance (HOMA-IR) method using the product of fasting insulin and FBG divided by 405.

All patients were followed up weekly by the clinical pharmacist in charge through patient encounters to ensure compliance with the treatment regimen and assess any adverse effects.

Outcomes

Primary outcomes

The patients improved lipid profiles, liver function tests, glycaemic control measures, and vitamin D levels at the end of the research.

Secondary outcomes

The decrease in the degree of steatosis in the US with the reduction in hs-CRP, NO, MDA, hepassocin, STAT-3, and TNF- α at the end of the study.

Sample Size Calculation

Based on data from prior research²⁸, the sample size was calculated using serum LDL-C as the primary dependent variable, a type I error of 0.05, and a study power of 85%. We concluded with the sample size of 50 patients in each group based on the recommended formula for parallel clinical trials, taking into consideration the dropout rate.

Statistical Analysis

The statistical analysis was carried out using GraphPad Prism version 7 (GraphPadsoftware, Inc, San Diego, CA, USA). To identify significant differences within groups before and after the intervention, the Paired Student's *t*-test was used. An unpaired Student's *t*-test was used to evaluate significant differences between groups before and after the intervention. The data was presented as the mean \pm standard deviation (SD). For categorical data statistical analysis, the Chi-square test was used. All *p*-values were two-tailed, with (*p* < 0.05) considered statistically significant.

Results

Clinical and Demographic Data in the Two Study Groups

This study involved 100 NAFLD patients who were randomly assigned to groups 1 or 2. For four months, group one received standard treatment

Table I. Clinical and demographic data in the two study groups.

Parameter	Group (1)	Group (2)	p-value
Age (year)	42.11 ± 13.97	46.06 ± 16.95	0.206
Sex (M/F)	26/24	24/26	0.747
Height (m ²)	1.702 ± 0.108	1.696 ± 0.098	0.671

Data are expressed as mean ± SD; M: Male, F: Female; Significance at ($p < 0.05$). Group 1: 50 patients received the standard conventional therapy in addition to a placebo for 4 months. Group 2: 50 patients were given the standard conventional therapy plus cholecalciferol as a high oral loading dose of 300,000 IU followed by a daily oral dose of 800 IU for 4 months.

plus a placebo; for four months, group two received standard conventional therapy plus cholecalciferol administered as a high oral loading dose of 300,000 IU followed by a daily oral dose of 800 IU. Table I displays their baseline statistics. There were no significant differences in demographic data between the studied groups; age ($p = 0.206$), sex ($p = 0.747$), weight ($p = 0.790$), and height ($p = 0.671$).

Data for Anthropometric Measures in the Two Study Groups

Regarding group 1, Table II shows that there were no significant variations in all measured parameters in comparison to after treatment as follows: weight (76.66 ± 9.447 vs. 76.36 ± 9.035, $p = 0.365$), waist circumference (92.81 ± 16.99 vs. 93.77 ± 16, $p = 0.589$), and body mass index (26.50 ± 2.715 vs. 26.43 ± 2.851, $p = 0.547$) using paired t -test.

Regarding group 2, Table II shows that there were no significant variations in all measured parameters in comparison to after treatment as follows: weight (74.60 ± 9.311 vs. 74.50 ± 9.338, $p = 0.540$), waist circumference (92.81 ± 16.99 vs. 93.77 ± 16, $p = 0.589$), and body mass index (26.50 ± 2.715 vs. 26.43 ± 2.851, $p = 0.547$) using paired t -test.

Table II showed that comparisons between groups using the unpaired t -test revealed no significant differences in baseline values between the studied groups. All measured parameters showed no significant change after four months of intervention.

Glycaemic Index, Lipid Profile, Liver Function Tests, and Vitamin D Data in the Two Study Groups

Regarding group 1, Table III shows that there were no significant variations in all measured parameters in comparison to after treatment as follows: fasting insulin (11.30 ± 2.309 vs. 11.64 ± 2.705, $p = 0.094$), FBG (132.8 ± 15.99 vs. 132.5 ± 14.89, $p = 0.871$), HOMA-IR (3.709 ± 0.922 vs. 3.802 ± 0.943, $p = 0.330$), LDL (169.5 ± 20.87 vs. 170.9 ± 2,054, $p = 0.069$), TG (171.8 ± 20.28 vs. 170.5 ± 18.67, $p = 0.114$), TC (191.2 ± 21.14 vs. 192.5 ± 19.65, $p = 0.240$), HDL (45.14 ± 9.141 vs. 44.88 ± 9.147, $p = 0.640$), ALT (53.52 ± 11.39 vs. 52.80 ± 10.84, $p = 0.256$), AST (48.76 ± 11.90 vs. 49.06 ± 11.29, $p = 0.80$), albumin (4.980 ± 0.850 vs. 4.8 ± 0.814, $p = 0.219$), and vitamin D (18.55 ± 5.294 vs. 18.24 ± 4.788, $p = 0.759$) using paired t -test.

Regarding group 2, Table III shows that there were no significant variations in the following measured parameters in comparison to after treatment as follows: fasting insulin (10.87 ± 3.303 vs.

Table II. Data for anthropometric measures in the two study groups.

Parameter	Group 1			Group 2			^b p-value
	Before treatment	After treatment	^a p-value	Before treatment	After treatment	^a p-value	After treatment
Weight (kg)	76.66 ± 9.447	76.36 ± 9.035	0.365	74.60 ± 9.311	74.50 ± 9.338	0.540	0.313
WC (cm)	92.81 ± 16.99	93.77 ± 16	0.589	96.78 ± 14.98	97.29 ± 15.56	0.587	0.809
BMI (kg/m ²)	26.50 ± 2.715	26.43 ± 2.851	0.547	26.08 ± 3.537	26.05 ± 3.562	0.606	0.563

Data are represented as mean ± SD; WC: Waist circumference, BMI: Body mass index, Kg: Kilogram, cm: Centimetre, ^asignificance level within the same group by paired t -test. ^blevel of significance between groups using unpaired t -test. Significance at ($p < 0.05$). Group 1: 50 patients received the standard conventional therapy in addition to a placebo for 4 months. Group 2: 50 patients were given the standard conventional therapy plus cholecalciferol as a high oral loading dose of 300,000 IU followed by a daily oral dose of 800 IU for 4 months.

Table III. Glycaemic index, lipid profile, liver function tests, and Vitamin D data in the two study groups.

Parameter	Group 1			Group 2			^b <i>p</i> -value
	Before treatment	After treatment	^a <i>p</i> -value	Before treatment	After treatment	^a <i>p</i> -value	After treatment
Fasting insulin (mU/L)	11.30 ± 2.309	11.64 ± 2.705	0.094	10.87 ± 3.303	73.44 ± 2.535	0.997	0.145
FBG (mg/dl)	132.8 ± 15.99	132.5 ± 14.89	0.871	133.5 ± 16.86	134.5 ± 14.47	0.574	0.809
HOMA-IR	3.709 ± 0.922	3.802 ± 0.943	0.330	3.543 ± 0.956	3.599 ± 0.879	0.759	0.269
LDL (mg/dl)	169.5 ± 20.87	170.9 ± 20.54	0.069	171.1 ± 21.54	161.7 ± 19.81	0.0001	0.024
TG (mg/dl)	171.8 ± 20.28	170.5 ± 18.67	0.114	170.9 ± 18.35	162.8 ± 17.35	0.0001	0.036
TC (mg/dl)	191.2 ± 21.14	192.5 ± 19.65	0.240	192.7 ± 21.44	183.5 ± 20.24	0.0001	0.025
HDL (mg/dl)	45.14 ± 9.141	44.88 ± 9.147	0.640	45.04 ± 8.685	48.6 ± 8.207	0.0001	0.034
ALT (U/L)	53.52 ± 11.39	52.80 ± 10.84	0.256	52.92 ± 11.46	47.52 ± 11.60	0.0001	0.0207
AST (U/L)	48.76 ± 11.90	49.06 ± 11.29	0.80	44.62 ± 11.08	45.20 ± 10.78	0.391	0.0835
Albumin (g/dl)	4.980 ± 0.850	4.8 ± 0.814	0.219	4.9 ± 0.914	4.95 ± 0.893	0.790	0.382
Vitamin D (ng/ml)	18.55 ± 5.294	18.24 ± 4.788	0.759	18.38 ± 4.911	37.18 ± 7.966	0.001	0.001

Data are represented as mean ± SD; WC: Waist circumference, BMI: Body mass index, Kg: Kilogram, cm: Centimetre, ^asignificance level within the same group by paired *t*-test. ^blevel of significance between groups using unpaired *t*-test. Significance at (*p* < 0.05). Group 1: 50 patients received the standard conventional therapy in addition to a placebo for 4 months. Group 2: 50 patients were given the standard conventional therapy plus cholecalciferol as a high oral loading dose of 300,000 IU followed by a daily oral dose of 800 IU for 4 months.

73.44 ± 2.535, *p* = 0. 0997), FBG (133.5 ± 16.86 vs. 134.5 ± 14.47, *p* = 0.574), HOMA-IR (3.543 ± 0.956 vs. 3.599 ± 0.879, *p* = 0.759), AST (44.62 ± 11.08 vs. 45.20 ± 10.78, *p* = 0.391), and albumin (4.9 ± 0.914 vs. 4.95 ± 0.893, *p* = 0.790). However, there was a statistically significant change in the following parameters when compared to after treatment as follow: LDL (171.1 ± 21.54 vs. 161.7 ± 19.81, *p* = 0.0001), TG (170.9 ± 18.35 vs. 162.8 ± 17.35, *p* = 0.0001), TC (192.7 ± 21.44 vs. 183.5 ± 20.24, *p* = 0.0001), HDL (45.04 ± 8.685 vs. 48.6 ± 8.207, *p* = 0.0001), ALT (52.92 ± 11.46 vs. 47.52 ± 11.60, *p* = 0.0001), and vitamin D (18.38 ± 4.911 vs. 37.18 ± 7.966, *p* = 0.001) using the paired student *t*-test.

Table III shows no significant difference in baseline values between the studied groups using the unpaired *t*-test. After four months of intervention, there was no substantial change in the following measured parameters, fasting insulin (*p* = 0.145), FBG (*p* = 0.809), HOMA-IR (*p* = 0.269), AST (*p* = 0.0835), and albumin (*p* = 0.382). However, there were statistically significant changes in the following parameters when compared to after treatment, as follows: LDL (*p* = 0.024), TG (*p* = 0.036), TC (*p* = 0.025), HDL (*p* = 0.034), ALT (*p* = 0.0207), and vitamin D (*p* = 0.001).

Regarding serum vitamin D, all patients had sub-normal vitamin D levels, i.e., mean serum 25(OH)D < 30 ng/ml. In group 1 (placebo), 29 and 21 patients were vitamin D deficient and insufficient, respectively. While in group 2 (treatment group), 30 and 20

patients were vitamin D deficient and insufficient, respectively. At the end of the study, 42 patients in group 2 became vitamin D sufficient, 8 were found to be vitamin D insufficient, and no patient was found in the vitamin D deficient category. However, no significant difference was observed in the group 1 compared to their baseline categories.

Analysis of Inflammatory And Oxidative Stress Biomarkers In The Two Study Groups

Regarding group 1, Table IV shows that there were no significant differences in all measured parameters in comparison to after treatment as follows: TNF-α (368.3 ± 13.90 vs. 368.1 ± 19.08, *p* = 0.949), hs-CRP (10.41 ± 2.769 vs. 11.08 ± 2.290, *p* = 0.170), STAT-3 (93.22 ± 9.04 vs. 93.04 ± 6.709, *p* = 0.867), MD (65.73 ± 11.96 vs. 64.89 ± 13.04, *p* = 0.238), NO (281.4 ± 10.32 vs. 280.8 ± 9.562, *p* = 0.134), and hepassocin (9.232 ± 0.5098 vs. 9.147 ± 0.4452, *p* = 0.275) using paired *t*-test.

Regarding group 2, Table IV shows that there were statistically significant differences in all measured parameters in comparison to after treatment apart from TNF-α as follows: TNF-α (367.6 ± 20.22 vs. 367.5 ± 20.09, *p* = 0.992), hs-CRP (9.475 ± 2.550 vs. 8.386 ± 2.385, *p* = 0.0008), STAT-3 (94.68 ± 7.306 vs. 79.91 ± 4.814, *p* = 0.0001), MD (59.52 ± 9.019 vs. 58.52 ± 9.251, *p* = 0.017), NO (279.0 ± 10.52 vs. 144.7 ± 65.68, *p* = 0.0001), and hepassocin (8.439 ± 0.8322 vs. 8.043 ± 1.053, *p* = 0.0004) using paired *t*-test.

Table IV. Analysis of inflammatory and oxidative stress biomarkers in the two study groups.

Parameter	Group 1			Group 2			^b p-value
	Before treatment	After treatment	^a p-value	Before treatment	After treatment	^a p-value	After treatment
TNF- α (pg/ml)	368.3 \pm 13.90	368.1 \pm 19.08	0.949	367.6 \pm 20.22	367.5 \pm 20.09	0.992	0.890
hs-CRP (mg/L)	10.41 \pm 2.769	11.08 \pm 2.290	0.170	9.475 \pm 2.550	8.386 \pm 2.385	0.008	0.0001
STAT-3 (pg/ml)	93.22 \pm 9.04	93.04 \pm 6.709	0.867	94.68 \pm 7.306	79.91 \pm 4.814	0.0001	0.0001
MDA (μ mol/L)	65.73 \pm 11.96	64.89 \pm 13.04	0.238	59.52 \pm 9.019	58.52 \pm 9.251	0.017	0.0126
NO (μ mol/L)	281.4 \pm 10.32	280.8 \pm 9.562	0.134	279.0 \pm 10.52	144.7 \pm 65.68	0.0001	0.0001
Hepassocin (ng/ml)	9.232 \pm 0.5098	9.147 \pm 0.4452	0.275	8.439 \pm 0.8322	8.043 \pm 1.053	0.0004	0.0001

Data are represented as mean \pm SD, hs-CRP: High sensitive C- reactive protein, TNF- α : Tumor necrosis factor alpha, STAT-3: signal transducer and activator of factor-3, NO: Nitric oxide, MDA: Malondialdehyde, pg/ml: Picograms/milliliter, ng/ml: Nanograms/milliliter, mg/l: Milligrams/deciliter, μ mol/L: micromole/liter. ^alevel of significance within groups by paired *t*-test. ^blevel of significance between groups using unpaired *t*-test. Significance at ($p < 0.05$). Group 1: 50 patients received the standard conventional therapy and placebo for 4 months. Group 2: 50 patients were given the standard conventional therapy plus cholecalciferol as a high oral loading dose of 300,000 IU followed by a daily oral dose of 800 IU for 4 months.

Table IV demonstrates no significant difference in baseline values between groups when comparing them using an unpaired *t*-test. Apart from TNF- α , there were statistically significant changes in all studied markers after four months of intervention, as follows: TNF- α ($p = 0.890$), hs-CRP ($p = 0.0001$), STAT-3 ($p = 0.0001$), MD ($p = 0.0001$), NO ($p = 0.0001$), and hepassocin ($p = 0.0001$).

Comparison of the Number of Patients in Both Groups in Different Liver Steatosis Grades at Baseline and the End of the Study

Regarding group 1, Figure 2 shows that there was no significant variation in the degree of steatosis as follows: mild degree (12 patients vs. 15), moderate degree (27 patients vs. 26), and severe degree (11 patients vs. 9).

Regarding group 2, Figure 2 shows that there was no significant variation in the degree of steatosis as follows: mild degree (13 patients vs. 15), moderate degree (28 patients vs. 27), and severe degree (9 patients vs. 8).

None of the patients in group 2 reported any side effects after 4 months of daily cholecalciferol administration.

Discussion

Globally, non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver diseases affecting the population. In the absence of alcohol consumption, it is described as the build-up of fat in the hepatocytes that results in impaired liver function^{29,30}. Several tissues have

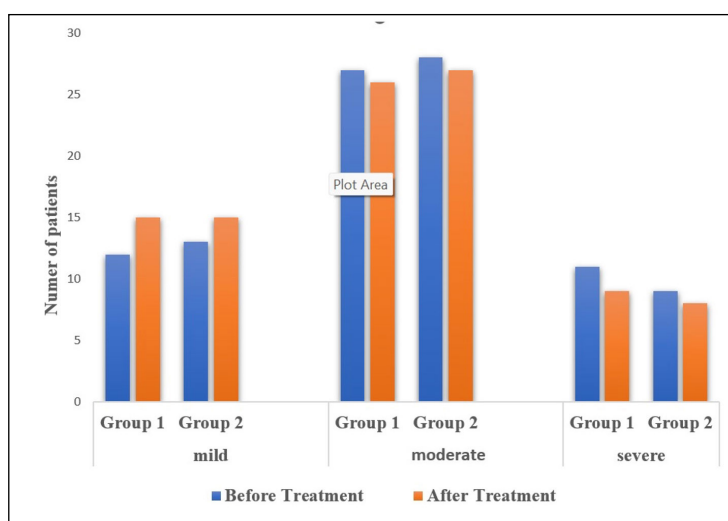


Figure 2. Comparison of the number of patients in both groups in different liver steatosis grades at baseline and the end of the study.

receptors for vitamin D, which is a fat-soluble vitamin. The genes controlling oxidative stress, inflammation, and the regulation of the liver's lipid and glucose metabolism are all impacted by vitamin D receptors³¹. A previous study³³ on rats showed that decreased vitamin D intake is related to NAFLD development and progression through pathways involving oxidative stress and inflammation³². A strong relationship between the stage and severity of NAFLD with low serum vitamin D levels was observed in several studies³¹⁻³³.

To our knowledge, this is the first randomized, placebo-controlled, double-blind study evaluating the effect of a high oral loading dose of vitamin D in NAFLD patients and its effect on serum hepassocin and STAT-3 followed by a daily maintenance dose of vitamin D for 4 months. The current study did not observe significant differences in serum TNF- α levels between the study groups. These findings were consistent with another research³⁴ that evaluated the impact of vitamin D on TNF- α in NAFLD patients. In another study³⁵, oral supplementation with 40,000 IU vitamin D₃ to overweight and obese subjects did not affect serum TNF- α levels after 1 year of supplementation. However, administering a single bolus dose (300,000 IU) of vitamin D₂ in vitamin D-deficient subjects significantly increased serum TNF- α after 3 months³⁶. These contradictory findings could be attributed to various diseases, subjects, vitamin D supplementation types, and doses, follow-up time, and other unknown cellular mechanisms between vitamin D and inflammatory cytokines. More research in this field will shed light on more facts.

We observed that correcting vitamin D levels in NAFLD patients could lower serum MDA levels and enhance antioxidant capacity by down-regulating NO synthase activity, indicating yet another mechanism by which vitamin D may improve NAFLD. These findings are in line with other studies^{34,37}. MDA, a lipid peroxidation marker, is proven to increase in NAFLD patients³⁸. Wiseman³⁹, the first to observe vitamin D's antioxidant action, discovered that vitamin D₃ and its active form 1,25(OH)₂D₃ inhibited iron-dependent liposomal lipid peroxidation. The oxidative stress markers depend on vitamin D levels, which change the intracellular signaling pathway and increase inflammation and liver injury³⁷. Vitamin D₃ inhibits the expression of inducible nitric oxide synthase in a rat's central nervous system during experimental allergic encephalomyelitis⁴⁰. By contrast, many former experimental findings

suggested that vitamin D may improve the activity and expression of endothelial nitric oxide synthase, which is critical for NO^{41,42}. The structural similarity between vitamin D compounds and cholesterol and ergosterol may be related to antioxidant capacity. In accordance with our findings, giving 300,000 IU of vitamin D₃ monthly for three months to vitamin D-deficient subjects could substantially reduce serum MDA levels⁴³. Another research⁴⁴ revealed that 1,25(OH)₂D₃ could lower MDA levels in rats with hepatocellular carcinoma.

When comparing the vitamin D group to the baseline and control groups, our findings showed a statistically significant decrease in serum STAT-3 levels. It is well known that cytokine increases in NAFLD patients are caused by reactive oxygen and nitrogen species (ROS and RNS) and byproducts of lipid peroxidation⁴⁵. Furthermore, oxidative stress in NAFLD promotes signal transduction and activation of transcription pathways (STAT-3) that induce hepatic damage and disease development up to its malignant result^{46,47}. Clearly, vitamin D directly suppresses oxidative stress biomarkers such as MDA, and other inflammatory cytokines such as IL-6, verified by the current study and another study⁴⁸. Vitamin D diminishes the gene transcription of nuclear factor kappa B cells (NF- κ B) with negative feedback on the IL-6/STAT-3 pathway⁴⁹. There were cross-signaling pathways between IL-6 and STAT-3 in several models of inflammation^{50,51}. Furthermore, inhibition of STAT-3 may occur due to the up-regulation of cytokine signalling-3 suppressor (SOCS-3) by adipose tissue or hepatocyte-derived inflammatory cytokine⁵¹. By these indirect mechanisms, "inflammation and oxidative stress suppression", vitamin D could reduce STAT-3 levels.

The current study revealed that vitamin D significantly reduced serum hepassocin compared to the baseline and control groups. This result came in accordance with other studies^{22,52}. Free fatty acids induce lipid building up in the liver and increase proinflammatory cytokines, including IL-6, which further activates STAT-3 to induce hepatic fibrosis⁵³. In addition to inflammation, hyperlipidemia induces the expression of hepassocin in primary hepatocytes⁵⁴. STAT-3 and hepatocyte nuclear factor-1 (HNF1) binding sites are found in the promoter region hepassocin, and IL-6 significantly induces the promoter activity of hepassocin, depending on the HNF1-binding site²⁰. Following the current research, vitamin D significantly reduced STAT-3 levels and de-

creased lipids accumulation, which may also indirectly decrease serum hepassocin.

It is well known that NAFLD patients have significantly higher hs-CRP serum levels when compared to healthy subjects⁵⁵. Previous research⁵⁶ found that hs-CRP is related to histopathologic changes in NAFLD that are independent of other risk factors. Suppressing serum hs-CRP levels would thus be an essential way to slow NAFLD progression and reduce the risk of cardiovascular disease, or at the very least, to comply with the therapy plan⁵⁷. We reported a significant decline in hs-CRP serum level, which was also reported by Foroughi et al⁵⁸, and Sharifi et al³⁴. In contrast, the vitamin D therapy regimen introduced by Barchetta et al⁵⁹ and Sakpal et al⁶⁰ did not significantly affect hs-CRP levels. According to previous research⁶¹, 1,25(OH)2D suppresses T-helper1 and may increase T-helper2, reducing inflammatory cytokine production and causing anti-inflammatory effects. Hepatic macrophages express not only VDR but also the enzyme 1-hydroxylase, resulting in the local synthesis of 1,25(OH)2D⁶². In agreement with our results, T2DM patients who received vitamin D-fortified drinks were compared with those who received unfortified drinks for 12 weeks, and significantly lower serum hs-CRP levels were detected in the former group⁶³.

The present study showed that vitamin D dosing significantly decreased serum ALT after 4 months of administration, but no significant changes were seen in other liver enzyme biomarkers. This was also shown by Sakpal et al⁶⁰, who found that the serum level of ALT decreased after a single 600,000 IU vitamin D shot supplementation along with the standard management for 6 months. This finding was also supported by Amiri et al⁶⁴, who demonstrated that ALT significantly reduced after administering a small dose of about 25µg calcitriol (~1,000 IU) along with a high caloric diet for 3 months. In contrast, other studies^{34,65} reported no significant effects on ALT. Some cross-sectional studies⁶⁶⁻⁶⁸ reported a significant association between 25(OH)D serum levels and liver enzymes. After controlling for confounding variables, a recent population study (n = 2,649)⁶⁹ found that having a high level of liver enzymes was linked with lower vitamin D levels. This result, however, was not statistically significant.

Furthermore, our study found that vitamin D positively impacts the lipid profile, with a significant decrease in serum TG, LDL, and TC and a significant increase in HDL levels. This finding agrees with Amiri et al's⁶⁵ and Sharifi et al's³⁴ re-

sults that confirmed the reducing effect of vitamin D on TG, TC, and LDL serum levels. Possible mechanisms for these effects include vitamin D's involvement in increasing lipoprotein lipase activity in adipose tissue⁷⁰. Increased vitamin D levels may also be linked to lower serum parathyroid hormone levels; *in-vitro* experiments have shown that parathyroid hormone can reduce lipolysis⁷¹. Furthermore, Carmeliet et al⁷² demonstrated that Vitamin D regulates calcium homeostasis by decreasing hepatic TG secretion, most likely by increasing calcium levels⁷². Calcium also combines with bile acids, facilitating the combination's fecal excretion. The greater the bile acid loss in feces, the greater the demand for new bile acids generated by the liver, and the lower serum cholesterol levels. As a result, vitamin D may lower cholesterol, triglyceride, and LDL levels by enhancing calcium absorption^{72,73}. Other investigations^{18,74}, however, have found no significant link between vitamin D levels in NAFLD patients and abnormalities in the lipid profile.

Furthermore, concerning the glycaemic index and anthropometric parameters, our result did not reveal any beneficial effect of vitamin D on all these parameters at the end of the study. Likewise, previous trials did not report any significant changes in the anthropometric measures^{34,58}, but some studies showed controversial effects on the glycaemic index and insulin sensitivity^{75,76}. Some theories were proposed in the literature to understand the reason behind these conflicting results. Among the discussed reasons, it appears that the period of vitamin D supplementation and IR measuring techniques are the most prevalent in our study's failure to document vitamin D effects on IR. To calculate IR, we used blood glucose and insulin levels. The euglycemic clamp technique or glucose tolerance testing may be more useful to properly measure insulin sensitivity.

Furthermore, based on von Hurst et al⁷⁷ research findings, a longer treatment period may be required. South Asian women were given 4,000 IU/d of vitamin D3 for 6 months during this research. Despite the elevated 25(OH)D serum concentration after 3 months of therapy, they determined no meaningful improvement in insulin sensitivity until after 6 months⁷⁷. Conversely, two studies^{51,58} revealed a substantial decline in FBG and HOMA-IR.

Further, at the end of the study, no change in the degree of liver steatosis in the US was found in both group. Similarly, Barchetta et al⁵⁹ did not report a significant difference in the fat fraction

measured by magnetic resonance after 24 weeks of oral high-dose vitamin D supplementation in T2DM patients with NAFLD⁵⁹. In contrast to our findings, El Amrousy et al⁶⁸ reported a significant improvement in hepatic steatosis and lobular inflammation by liver biopsy in the vitamin D group. A possible explanation for these differences may be attributed to different doses of vitamin D, study duration, and study populations.

Limitations

Although the study measures serum STAT-3 and hepassocin markers for the first time in NAFLD patients treated with Vitamin D, our study has two main limitations. Firstly, the lack of liver biopsy due to financial constraints hindered the follow-up of the histological changes in the liver. Secondly, the relatively short duration of the study. Further studies with a more extended period evaluating the histological changes in the liver with vitamin D loading and maintenance supplementation plans are required.

Conclusions

In conclusion, our randomized placebo-controlled trial demonstrated that hypovitaminosis D is common in NAFLD patients. High oral loading dose followed by daily oral doses of vitamin D had beneficial effects on serum ALT levels, hs-CRP levels, oxidative stress, STAT-3, hepassocin, and lipid profile of NAFLD patients.

Ethics Approval

The study protocol was revised and approved for scientific and ethical issues by the Institution Review Board of the Faculty of Medicine, Tanta University (approval code (35927/10/2022)).

Informed Consent

Informed consent was obtained from all patients included in the study.

Data Availability

Due to privacy/ethical restrictions, data are available on request.

Authors' Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critical-

ly reviewing the article. All authors gave final approval of the version to be published and have agreed on the journal to which the article has been submitted; they agree to be accountable for all aspects of the work.

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Conflicts of Interest

The authors declare no conflict of interest.

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