Effect of a moderately carbohydrate-restricted diet on liver enzymes, steatosis and fibrosis in normal-weight individuals with non-alcoholic fatty liver disease: study protocol for a parallel randomised controlled clinical trial

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ABSTRACT
Introduction Non-alcoholic fatty liver disease (NAFLD) is a hepatic condition that is considerably prevalent across the world. Dietary intakes, in which macronutrient composition is precisely planned, might be able to reduce inflammation, steatosis and fibrosis among patients with NAFLD. A moderately carbohydrate restricted diet with weight loss has been demonstrated to improve liver fat content among overweight or obese patients. However, there is no information about the appropriateness of such a restriction, without weight loss, in normal-weight patients. This randomised clinical trial will be aimed at assessing the effect of moderate carbohydrate restriction on liver enzymes, liver steatosis and fibrosis in normal-weight patients with NAFLD.

Methods and analysis This randomised controlled clinical trial will be conducted to evaluate the impact of a moderately carbohydrate restricted diet on liver enzymes, steatosis and fibrosis in 52 eligible normal-weight individuals with NAFLD. Transient elastography and controlled attenuation parameter with FibroScan will be applied to diagnose NAFLD. After individual matching based on body mass index, age and sex, patients will be randomly assigned to receive a moderately carbohydrate restricted diet or an isocaloric diet without carbohydrate restriction for 12 weeks. The primary and secondary outcomes in this study will be liver function indices, including liver steatosis and fibrosis, metabolic parameters and anthropometric measures. All these variables will be assessed at study baseline and postintervention.

STRENGTHS AND LIMITATIONS OF THIS STUDY
⇒ As far as we know, this is the first clinical trial that will be examined the impact of a moderately carbohydrate-restricted diet on liver function indices and metabolic parameters in normal-weight individuals with non-alcoholic fatty liver disease (NAFLD).
⇒ This clinical trial will be done on normal-weight individuals who newly diagnosed NAFLD with FibroScan.
⇒ This intervention will not have a specific biomarker to assess compliance with a moderately carbohydrate-restricted diet.
⇒ The duration of this intervention might not seem enough to examine the effect of a moderately carbohydrate-restricted diet on liver fibrosis.

INTRODUCTION
Non-alcoholic fatty liver disease (NAFLD) has been defined as the presence of fat in the liver at the amount of equal or more than 5% of its weight without significant alcohol consumption.1 It is strongly associated with metabolic syndrome and diabetes and may lead to cirrhosis and hepatocellular carcinoma.2-5 In parallel to obesity epidemic, the incidence of fatty liver is increasing worldwide.6 In Iran, it has been estimated that at least 34% of adult population are affected.7 Fatty liver is not restricted to obese people. This condition is also seen among 12.1% of non-obese individuals.8 The main effective strategy for management of NAFLD is weight loss.9 Given the key role of dietary carbohydrates in the development of NAFLD, it seems that a moderate restriction of carbohydrates, even without inducing any significant weight loss, might improve the fat content of the liver in normal-weight individuals with NAFLD.10-12 Earlier evidence has shown the
efficacy of this dietary pattern in controlling inflammation and other metabolic abnormalities. In a cross-over clinical trial, Rajaie et al.13 administered a moderately restricted carbohydrate weight loss diet to patients with metabolic syndrome for 12 weeks and found that adherence to such dietary pattern resulted in reduced inflammatory biomarkers compared with fat restricted weight loss diet. Such beneficial effects of low-carbohydrate diet were also reported by other investigators. However, the beneficial effects in these studies might be attributed to weight loss.14 15 In a randomised clinical trial (RCT) carried out on 18 obese individuals with NAFLD, it was found that in a similar weight loss, low-carbohydrate diet was more efficient in decreasing intrahepatic triglycerides (TG) than low-calorie diet.15 However, this is not a consistently reported finding across studies. Some studies have reported that despite a similar weight reduction, fat content of liver was not significantly different comparing low-carbohydrate and high-carbohydrate diets followed about 11 weeks among individuals with insulin resistance.16 Given the role of dietary carbohydrates in inducing inflammation and oxidative stress and the role of these abnormalities in fatty liver, we hypothesised that restriction of dietary carbohydrates might help normal-weight individuals reducing the fat content of the liver without inducing weight loss.17–19 Due to a lack of earlier evidence in this regard as well as consumption of high-carbohydrate diets in the Middle East,20 we designed the current clinical trial to examine the effect of moderately carbohydrate-restricted diet on liver fat content, liver fibrosis and liver function-related enzyme levels among patients with NAFLD.

**Aims**

This RCT will be aimed to assess the effect of moderate carbohydrate restriction on liver enzymes, liver steatosis and fibrosis in normal-weight patients with NAFLD.

**METHODS AND ANALYSIS**

**Study design**

A diagram of the study design is shown in figure 1. A flow chart of the study process is demonstrated in figure 2. In the present parallel clinical trial, individuals will be recruited from private clinics in Tehran based on inclusion criteria. Enrolment of study participants will be done based on age, gender (male/female) and body mass index (BMI) (±2) in different blocks, such that the first patient will be recruited if he/she met the inclusion criteria. Then, the second person in that block will be chosen based on the above-mentioned variables. This will be continued until having 34 blocks, each block containing

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**Figure 1**  Diagram of the study design.
2 people who are matched in terms of sex, age and BMI, as mentioned above. After recruitment, participants will be on a 2-week run-in period in which we will collect required information about their lifestyle habits, dietary intakes and medical history. To examine usual dietary intakes in this period, we will use 24-hour dietary recalls. Participants will be asked to complete three consecutive 24-hour dietary recalls, which include two working days and one weekend. In addition, a questionnaire about dietary habits will also be completed during this period. To assess the usual physical activity of subjects, participants will be requested to record their physical activity for 3 days, including two working days and one weekend. Then, information about age, sex, education, history of diseases and hospitalisation, surgery, and history of taking drugs, supplements, and smoking will be obtained from all participants using a general questionnaire. All measurements, including anthropometric assessment and biochemical evaluation, liver steatosis, and fibrosis by FibroScan, will be performed at this stage. After all these measurements, participants will be randomly assigned to intervention and control groups. Random allocation will be done by a person who is unaware of the study and intervention. In each block, the first code, coming out of the vase, will be assigned to the intervention group and the second code to the control group. Individuals in the intervention and control groups will receive a moderately carbohydrate-restricted diet and an isocaloric normal macronutrient diet, respectively, for 12 weeks. A description of these diets has been provided below. To evaluate the adherence to the prescribed diets throughout the study, four 24-hour dietary recalls (two working days and two non-working days) will be taken from each individual every 3 weeks. Participants will also be monitored by phone every week during the study to remind them to adhere to the prescribed diets and to resolve problems they might face with their diet in both intervention and control groups. At the end of the intervention, all measurements will be repeated again. The current study was reported based on the Standard Protocol Items: Recommendations for Interventional Trials 2013 checklist.

Participants
This RCT will be done in non-obese patients with NAFLD in Tehran, Iran. Patients will be recruited from the private clinics or general hospitals affiliated with Tehran University of Medical Sciences (TUMS), Tehran, Iran. Inclusion criteria in this study would be as follows: patients aged 18–65 with a BMI of between 18.5 and <25 kg/m², who have been diagnosed with NAFLD based on FibroScan findings by a gastroenterologist.

Non-inclusion criteria
Patients who are consuming alcohol, using drug or tobacco, or who are pregnant, breast feeding as well as individuals who are suffering from other liver diseases, smokers, individuals who are taking corticosteroids (including prednisolone, hydrocortisone, betamethasone) and those following weight loss diet during the last 3 months.
All participants will be informed about the study design and they have to provide written consent before enrolment in the study (online supplemental section A). The study is approved by the Bioethics Committee of TUMS, Tehran, Iran (IR.TUMS.MEDICINE.REC.1400.116). This clinical trial was registered in the IRCT website (IRCT20210119050086N1).

Intervention
To administer a moderately carbohydrate-restricted diet, we will compute energy requirements of each study participant. To do this, the basic metabolic rate of each subject will be calculated according to participants’ age, gender, height and current weight using the Harris-Benedict formula. Then, considering participants’ physical activity and the thermic effect of foods, total energy expenditure will be computed. In the moderately carbohydrate-restricted diets, the distribution of macronutrients for each participant will be done as follows: 40%–45% of energy from carbohydrates, 35%–40% of energy from fats and the remaining from proteins. Given the high fat content of this diet and the probable concerns this might raise for subjects, they will be requested to meet such an additional intake of fat from food sources of monounsaturated fatty acids and polyunsaturated fatty acids. Individuals in the control group will be received a diet that consists of 50%–55% of energy from carbohydrates, 25%–30% of energy from fats and the remaining from proteins. It should be noted that the aim of this study is the effect of carbohydrate composition on liver function; nevertheless, we will consider 5% of carbohydrate energy from simple carbohydrate and the remaining from complex carbohydrate in both intervention and control group. All dietary menus will be divided into six daily meals, including three main meals and three snacks. We will also provide food exchange lists and other resources of information on dietary macronutrient compositions and calories, to help each individual to make food choices of their preference.

Sample size
The sample size in this study was computed using hepatic steatosis as the key variable. Considering type I error of 5% (α<0.05), the study power of 90% and the expected mean difference in hepatic steatosis as the primary outcome between the intervention and control group, we will need 22 subjects in each group and 44 subjects in total. To consider a possible drop-out of 20%, we will enrol a sample of 52 participants in each group according to our inclusion criteria.

Study outcomes
The primary outcomes of this clinical trial will be measurement of serum liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT), as well as determining level of steatosis by FibroScan. The major secondary outcome variables will be determining liver fibrosis stage by FibroScan, lipid profile including serum total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL_C) and low-density lipoprotein cholesterol (LDL_C), fasting blood sugar, fasting insulin, Homeostatic model Assessment for Insulin Resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) and anthropometric indicators including weight, height and waist circumference (WC). All these measurements will be assessed at preintervention and postintervention phases.

Assessment of dietary intake and physical activity
To assess dietary intake of each participant, a 1-day 24-hour dietary recall will be taken by an experienced nutritionist at weeks 3, 6, 9 and 12 during the study. These days will be distributed throughout the week to include two working days and two weekends. The format of dietary recalls is shown in online supplemental section B. Individuals will be asked to report their daily dietary intake based on household measures. Then, the average nutrient intake of study participants will be calculated based on the grams of consumed foods they have reported. For this computation, Nutritionist IV software, the database of which is modified based on Iranian foods, will be used.

To be sure of no significant change in physical activity level throughout the study, we will examine participants’ activity for 4 days including two working days and two non-working days at weeks 3, 6, 9 and 12 of intervention. To evaluate (monitor) the physical activity level of individuals, an International Physical Activity Questionnaire-Short Form which was previously validated in Iran will be used. Participants will be educated how to record their type, time spent and intensity of each physical activity during the study. Results of the average activity of subjects will be expressed as metabolic equivalent per hour per day considering the available published guidelines. The format of physical activity record is presented in online supplemental section C.

Assessment of hepatic steatosis and fibrosis
Liver fibrosis and steatosis in study participants will be evaluated by transient elastography and controlled attenuation parameter (CAP), respectively with FibroScan at preintervention and postintervention phases through the same gastroenterologist. FibroScan is a non-invasive device for assessment of liver stiffness and steatosis. To evaluate liver stiffness, a transient elastography with FibroScan will be used. Patients will be asked to fast for at least 2 hours before the test. Measurements will be performed with the probe. The probe will be placed on the intercostal space and right lobe of the liver of participants lying in the dorsal decubitus position. FibroScan will be taken in 10 min. The unit of liver stiffness will be expressed as kilopascals (Kpa). To examine liver steatosis, a CAP test of the FibroScan device will be used. The results will be reported in decibels per metre (dB/m). The categorisation of people in terms of CAP will be done based on the below-mentioned cut-offs.
CAP results will vary between 100 and 400 dB/m. The cut-offs for CAP will be:
S0: CAP><230.
S1: 230<CAP>275.
S2: 275<CAP<300.
S3: CAP>300.

Biochemical assessment
Biochemical factors will be measured in the laboratory of TUMS. After 12 hours fasting, a 10cc blood sample will be taken from each patient at preintervention and postintervention phases. The sample will be centrifuged at 3000 rpm for 10 min to extract serum. The level of liver enzymes (ALT, AST) and serum lipid profile (TC, TG, LDL-C and HDL-C) will be measured by the enzymatic colorimetric method using commercial kits (Pars Azmoon kit, Tehran, Iran). Serum concentrations of GGT will also be measured using the calorimetric-kinetic method proposed by the International Federation of Clinical Chemistry. The serum level of fasting blood glucose will be measured by glucose oxidase using a glucose specific kit. Serum fasting insulin concentrations will be examined using a commercial ELISA kit (IBT, USA). In addition, the following formulas will be applied to calculate QUICKI and HOMA-IR, respectively:

\[
\text{QUICKI} = 1/ (\log \text{ (fasting insulin } \mu U/mL) + \log \text{ (fasting glucose } mg/dL). \\
\text{HOMA-IR} = (\text{fasting insulin } (\mu U/L) \times \text{ fasting glucose } (mmol/L))/22.5.
\]

Assessment of other variables
Anthropometric measures including weight, height and WC will be measured using standard protocols. BMI will be calculated by dividing weight (kg) by squared height (m²). In addition, a general questionnaire to gather information on participants’ age, sex, educational status, marital status, smoking, medical history, supplement use, occupation, medication use and menopausal status will be used in the current study.

Statistical analysis
Normality of variables’ distribution will be assessed using the Kolmogorov-Smirnov test. If a log-transformed variable has a normal distribution, its log will be used in the analysis. The findings will be presented as mean (SD) for numerical data, frequency (percentage) for categorical variables, and median (25th, 75th) for values with skewed distribution. The independent samples t-test will be applied to compare the means (SD) of normally distributed variables between the two groups. The Mann-Whitney U test will be used for values with skewed distribution, and the median (25th, 75th) will be reported in such conditions. To do a within-group comparison, we will use the paired-sample t-test and Wilcoxon signed-rank test for values with normal and non-normal distribution, respectively. We will use the χ² test and Fisher’s exact test to examine differences in qualitative variables between the two groups. Multivariate analysis of covariance, as a general linear model, will be used to examine the effects of a carbohydrate-restricted diet on outcome variables. In this analysis, baseline values of outcome variables and potential confounding variables, which are different between the intervention and control groups, will be adjusted to avoid potential risk of bias and detect independent results. Statistical analysis was carried out using SPSS statistical software (SPSS, V.26). A p<0.05 will be considered as statistically significant.

Ethics and dissemination
The present clinical trial study was accepted by the ethics committee of TUMS (Tehran University of Medical Sciences) (code: IR.TUMS.MEDICINE.REC.1400.116). Moreover, this study was registered in the Iranian Registry of Clinical Trials (www.irct.ir) at 20 February 2021 (code: IRCT20210119050086N1).

Patient and public involvement
Patients and/or the public were not involved in the design, conduct reporting or dissemination plans of this study.

Trial status
The recruitment will be started on December 2021.

DISCUSSION
NAFLD, being a major public health problem with an increasing prevalence worldwide, can lead to fibrosis, cirrhosis and sometimes liver carcinoma. Besides weight loss, there is no consensus about the appropriate strategy to manage patients with NAFLD. Weight loss has been recommended for overweight or obese patients with NAFLD, but there is no information about the appropriate method for managing NAFLD among normal-weight patients. One in every eight individuals with NAFLD has a normal BMI. Although the aetiology of NAFLD is not clear, genetics is known as a crucial factor in the pathogenesis of NAFLD in non-obese subjects. Emerging evidence suggests that PNPLA3 is more common in non-obese people with NAFLD. In addition, other studies have found that high fructose intake is linked to fatty liver disease via gut microbiota changes in NAFLD patients of normal weight. Given the importance of dietary carbohydrates in the aetiology of NAFLD, we hypothesised that restricting carbohydrate intake without reducing calorie intake might be an appropriate strategy in patients with normal weight. Although several studies have examined the effects of macronutrient manipulation among patients with NAFLD, we are aware of no study that evaluated the effects of a moderate carbohydrate-restricted diet, without weight loss, in normal-weight individuals with NAFLD. Recent published studies have found that adherence to a low or moderate carbohydrate diet improves inflammation, liver enzyme levels and liver fat content among overweight or obese patients with NAFLD.
None of the earlier studies have assessed the effect of macronutrient manipulation on liver steatosis and fibrosis. Findings from this investigation might be applicable to managing NAFLD patients with normal weight. Considering that dietary modification is almost the safest and cheapest method for disease management, our findings can be extensively used by nutritionists, dietitians, and gastroenterologists and hepatologists.

To the best of our knowledge, this is the first RCT that will assess the effects of a moderately carbohydrate-restricted diet on liver enzymes, liver steatosis, fibrosis, lipid profile and glycemic variables. Furthermore, the study would be included newly diagnosed normal-weight NAFLD patients whose conditions will be diagnosed using FibroScan. However, several limitations should be considered. First, there is no specific biomarker to examine compliance with moderate carbohydrate restriction, so we will assess compliance by questionnaires. Therefore, self-reporting of dietary intake might affect the study findings. Second, the duration of intervention in this study is designed to be 12 weeks, which might not seem enough time to observe the effect of dietary intervention on liver fibrosis. However, some published studies have found significant effects on liver fibrosis following dietary interventions for 3 months. Third, the gold standard for assessment of liver fat is MRI-PDFF. However, the use of FibroScan has also been proven to be a reliable and non-invasive method for the diagnosis of steatosis and fibrosis in patients with NAFLD. Finally, based on the ‘multihit’ theory, as gut microbiota exerts a crucial role in the pathogenesis and progression of NAFLD, unknown effects of them on study outcomes should be considered as an important limitation. Human subjects with different gut microbiota have limited abilities to digest complex dietary polysaccharides. Therefore, polysaccharides are fermented into short-chain fatty acids and monosaccharides by gut microbiota, which is a factor limiting the gut microbiota to hepatic steatosis.

**CONCLUSION**

In conclusion, findings from the current clinical trial can inform better management of NAFLD patients who have normal weight.

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**Contributors** DF, S-AK and AE contributed to conception and study design. AAS contributed to drafting proposal. All authors had a significant contribution in developing the study Methodology. FD, SEM and SMA contributed to data collection, data entry and data analysis. FD, SEM and AE drafted the manuscript. All authors had a significant contribution in finalising the first draft of the manuscript. AE supervised the whole study. All authors read and approved the final manuscript.

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**Competing interests** None declared.

**Patient and public involvement** Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

**Patient consent for publication** Not applicable.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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Supplementary file

A) "Consent Form"

I ........................................ hereby agree to participate in a research project entitled "The effect of moderately carbohydrate restricted diet on liver enzymes, steatosis and fibrosis in normal-weight individuals with non-alcoholic fatty liver disease: study protocol for a parallel randomized controlled clinical trial" under the supervision of Dr. Ahmad Esmaillzadeh and Mrs. Fatemeh Dashti.

It was explained to me about the effect of adherence to the moderate carbohydrate restriction diet on liver enzymes and steatosis will be studied.

In this research, I will answer the questions about my characteristics and 24-hour dietary recalls, and blood sample will be taken from me at the beginning and end of the intervention. The present study is designed to be 12 weeks. During the research period, I will be adherenced to the moderately restricted carbohydrate diet during the intervention.

My name and all information that is taken from me will be remained confidential (in writing) and the research results will be published as the general answer of the studied group and the individual results will be presented without mentioning names.

The researcher has answered all my questions, so I agree to participate in this research. By mentioning this, this agreement will not prevent legal actions - in case of illegal action or inhumane method.

Name and surname of the person being studied:
Study address and phone number:

Date and signature of the participant:

Statement of the research officer: I have informed the participant about the nature of the above plan process and the treatment used and the possible risks. I have answered all questions to the best of my ability. I will inform the participant of any changes in possible risks and benefits during the study or information that will depend on the participant's willingness to continue treatment in this study.
Supplementary file

B) Food recall

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C) Physical activity record

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